Universidade de Lisboa

Faculdade de Ciências

Departamento de Biologia Animal

Habitat use, growth and reproductive behaviour of the western ruivaco (Cyprinidae): contributes to the restocking of wild populations

Mestrado em Biologia da Conservação

Daniel Chagas Roquette Mameri

Dissertação orientada por:

Doutora Carla Sousa Santos (ISPA–MARE)

Doutora Maria Filomena Magalhães (FCUL–CE3C)

2015

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COLLABORATIONS

The following thesis was developed in collaboration with ISPA - University Institute, the Vasco da Gama Aquarium (Portuguese Navy) and the Campelo Station (Quercus), following the protocol established by these institutions regarding the project on the *ex situ* conservation of endangered Iberian cyprinids.

AGRADECIMENTOS

A realização desta tese só foi possível graças a um conjunto de pessoas que, de uma forma ou de outra, estiverem sempre presentes para me apoiar. Correndo o risco de me esquecer de alguém, não posso deixar de fazer aqui alguns agradecimentos.

À Doutora Carla Sousa Santos, por ter aceite orientar-me nesta tese, por me ter acolhido no projecto de conservação de ciprinídeos endémicos, pelo entusiasmo, dedicação e enorme paciência que sempre teve para me ensinar ao longo de toda a tese, pelas palavras de encorajamento e pelo seu enorme sentido prático aquando da realização do trabalho de campo…foi um prazer trabalhar contigo!

À Professora Maria Filomena Magalhães, por ter aceite ser minha orientadora interna, por me ter despertado o interesse em ecologia fluvial, pela a disponibilidade que revelou em, literalmente, todos os momentos da realização da tese, e por todo o optimismo que demonstrou quando o meu pessimismo se acentuava!

À equipa do Aquário Vasco da Gama, com um especial obrigado à Doutora Fátima Gil e ao Zé Pedro, pela simpatia com que sempre me receberam e pelo apoio que me deram na componente do comportamento reprodutor do ruivaco-do-Oeste.

À equipa da Estação Aquícola de Campelo, pela colaboração na monitorização das populações cativas e pela boa-disposição que demonstraram, quer nas monitorizações, quer nas acções de repovoamento.

Ao meu pai, pela companhia e por todos os sacrifícios que fez para que eu conseguisse estar aqui e seguir a área que sempre quis; à minha mãe, a quem devo muito daquilo que sou hoje…o meu percurso não seria o mesmo sem estas pessoas!

Por último…um especial obrigado a quem me acompanhou mais de perto ao longo destes 5 anos; é difícil resumir tudo num parágrafo, por isso digo “apenas”: muito obrigado por terem estado lá em todos os momentos, pela companhia bem cedo pela manhã, pelas gargalhadas à hora de almoço, pelos finais de tarde descontraídos, por não me terem deixado ir abaixo nos momentos mais difíceis, por me levarem a querer fazer sempre mais e melhor…por serem uma segunda família, numa faculdade que foi a minha segunda casa…com isto, resta-me apenas agradecer-vos e dizer-vos “até já”!

RESUMO

Os ciprinídeos (Cyprinidae) constituem a família mais numerosa de peixes de água doce, tendo uma distribuição a nível global e ocupando uma grande diversidade de nichos ecológicos. Na Península Ibérica, encontram-se representados por um grande número de espécies endémicas, a maioria das quais com estatutos de conservação elevados. Para além das ameaças inerentes aos ecossistemas fluviais (*e.g.* poluição, proliferação de espécies exóticas e destruição de habitats) e do carácter intermitente de grande parte dos rios da região, os ciprinídeos ibéricos apresentam, na sua grande maioria, populações fragmentadas e com reduzida diversidade genética, o que potencia o seu risco de extinção.

O ruivaco-do-Oeste *Achondrostoma occidentale* é uma das espécies endémicas de Portugal que se encontra fortemente ameaçada. Este pequeno ciprinídeo apresenta uma distribuição muito restrita, limitada às ribeiras do Alcabrichel, Sizandro e Safarujo, onde as populações já com baixa diversidade genética enfrentam ainda graves problemas de poluição das águas e destruição de habitats.

Face à situação de risco do ruivaco-do-Oeste e ao contexto de seca ocorrido em Portugal em 2006, iniciou-se em 2007 um programa de conservação *ex situ* desta espécie. Este programa permitiu até à data, a realização de sete acções de repovoamento das populações de ruivaco-do-Oeste do Alcabrichel, Sizandro e Safarujo, com a libertação de um total de 6577 indivíduos. Apesar do sucesso da reprodução do ruivaco-do-Oeste em cativeiro, a sua ecologia permanece pouco conhecida, sendo evidente a falta de informação de base sobre a distribuição e abundância das populações naturais e parâmetros de história de vida, nomeadamente no que se refere aos padrões de crescimento (tanto das populações selvagens como das criadas em cativeiro) e ao comportamento reprodutor. O esclarecimento destes vários aspectos reveste-se de extrema relevância para definir directrizes para futuras acções de repovoamento de populações selvagens desta espécie.

Neste contexto, e através de três abordagens distintas, pretende-se com esta tese: (1) avaliar o uso do habitat e o estado actual das populações selvagens, através de estimativas de abundância relativa e determinação do comprimento e condição física; (2) estudar o crescimento das populações em cativeiro e compará-lo com o das populações selvagens; (3) descrever o comportamento reprodutor da espécie, usando uma população mantida em cativeiro em condições que recriam o mais fielmente possível o ambiente natural. Com base

na análise integrada dos resultados obtidos com esta abordagem multidisciplinar, pretendem-se indicar formas de melhorar a eficácia das futuras acções de repovoamento a realizar no âmbito do programa de conservação *ex situ* de que esta espécie é alvo.

A monitorização das populações selvagens foi realizada entre Setembro e Outubro de 2014, no final da estação seca, quando a conectividade das ribeiras Alcabrichel, Sizandro e Safarujo estava ainda interrompida e os peixes se encontravam confinados em pêgos isolados, que funcionam como refúgios estivais e fontes de colonizadores para o sistema na estação húmida.

As zonas montante das três ribeiras foram percorridas de carro e mapearam-se os pêgos persistentes. Em cada um dos 14 pegos inventariados quantificaram-se, 15 variáveis de habitat, descritoras da morfologia do pêgo (*i.e.* comprimento, largura e profundidade máximas, área e volume), ensombramento das galerias ripícolas, abundância de macrófitas e de detritos, substrato dominante, heterogeneidade do substrato, temperatura da água, pH e concentração de amónia, nitritos e nitratos na água. A abundância relativa de *A. occidentale* foi estimada através de pesca eléctrica, sendo o tempo de amostragem proporcional ao volume do pêgo. Foram medidos o comprimento à furca e o peso de todos os indivíduos capturados, com vista à obtenção de distribuições de comprimento e determinação do factor de condição, respectivamente. Para os peixes com mais de 40 mm, foram ainda retiradas três escamas da terceira fiada de escamas abaixo da barbatana dorsal, para posterior determinação da idade e taxas de crescimento. Foi também verificada a presença, na barbatana dorsal, da cicatriz do corte efectuado aos indivíduos libertados nas acções de repovoamento, para reconhecimento da sua proveniência.

Por forma a comparar o crescimento das populações selvagens com o das populações cativas, foram recolhidas escamas em indivíduos das populações mantidas em cativeiro no Aquário Vasco da Gama e na Estação Aquícola de Campelo.

A idade de cada indivíduo foi determinada através da contagem, à lupa, das marcas anuais de crescimento. O comprimento à furca em cada idade foi estimado por retrocálculo, a partir das relações entre o raio total da escama e o comprimento à furca, e foram posteriormente determinados os incrementos anuais de comprimento.

A componente deste trabalho relativa ao estudo do comportamento reprodutor do ruivaco-do-Oeste foi realizada no Aquário Vasco da Gama, com uma população do Rio Safarujo fundada em 2011. Após um período de observação informal iniciado em Novembro de 2014, os primeiros indícios de início de actividade reprodutora no tanque experimental foram observados no princípio de Abril de 2015, tendo-se então iniciado a descrição dos comportamentos reprodutores e a construção do respectivo etograma.

Posteriormente, no início de Maio de 2015, procedeu-se à quantificação dos comportamentos reprodutores em função do substrato (plantas, meadas de lã e areão) e do período do dia (8h30-11h30, 11h30-14h30 e 15h00-18h00). Os valores de temperatura e a quantidade de luz incidente no tanque experimental a cada quatro horas foram registados automaticamente com um *data logger*.

A monitorização dos refúgios estivais revelou que os pêgos com maior área se encontravam no Rio Sizandro, enquanto que os do Rio Alcabrichel apresentavam um maior volume. O número de indivíduos capturados por pêgo foi superior no Rio Sizandro e inferior no Rio Safarujo. A abundância relativa do ruivaco-do-Oeste em cada pêgo apresentou uma associação positiva com a temperatura da água e a abundância de macrófitas e negativa com a profundidade. Os maiores valores de abundância relativa foram observados em dois pêgos que foram alvo de acções de repovoamento, um no Rio Alcabrichel e outro no Rio Sizandro, que foram alvo de acções de repovoamento em 2011 e 2013. Nestes pêgos, os indivíduos capturados apresentaram também valores relativamente altos de condição física.

As distribuições de comprimento à furca obtidas para as populações do Alcabrichel e do Sizandro revelaram um padrão bimodal, reflectindo muito provavelmente o efeito das acções de repovoamento no aumento das classes de idade mais jovens (0+ e 1+). Na população do Safarujo, a única que não tinha sido alvo de qualquer acção de repovoamento à data das amostragens, a distribuição de comprimento apresentou um carácter unimodal e uma maior variabilidade.

A maior longevidade foi registada em indivíduos criados em cativeiro (5+). Estes indivíduos tendem a apresentar crescimentos superiores aos indivíduos selvagens, nas populações do Alcabrichel e do Safarujo. Pelo contrário, nas populações do Sizandro, os indivíduos selvagens apresentaram maior crescimento, podendo esta diferença dever-se às menores temperaturas a que está sujeita a população do Sizandro mantida em cativeiro em Campelo.

Relativamente ao comportamento reprodutor do ruivaco-do-Oeste, foi possível observar comportamentos de pré-postura que envolvem, maioritariamente, a perseguição de fêmeas em pré-postura por parte dos machos. Verificou-se uma maior prevalência dos comportamentos reprodutores junto a plantas aquáticas, em comparação com a actividade observada junto aos outros substratos disponíveis (areão e meadas de lã), o que sugere que as plantas podem constituir o substrato preferencial para a desova nesta espécie.

Globalmente, os dados recolhidos através da abordagem multidisciplinar desenvolvida para o estudo do ruivaco-do-Oeste revelaram que (1) as acções de repovoamento podem efectivamente contribuir para o reforço das classes de idade mais jovens e para um possível aumento de tamanho e condição física individual; (2) pêgos com maior volume e abundância de macrófitas são locais potenciais para futuras acções de repovoamento; (3) a presença de plantas aquáticas é, muito provavelmente, essencial para o sucesso reprodutor desta espécie e consequentemente para o recrutamento de colonizadores em cada ano, sendo este um dos critérios que deve ser tido em conta na escolha de locais para futuras acções de repovoamento; (4) independentemente das boas indicações obtidas através das monitorizações de populações selvagens, o programa de conservação *ex situ* desta espécie, para ser plenamente conseguido, deverá ser acompanhado por uma melhoria significativa de troços de cada uma das ribeiras habitadas pelo ruivaco-do-Oeste.

ABSTRACT

Iberian cyprinids include a high number of endemic and highly threatened species. One of such species is the Portuguese western ruivaco *Achondrostoma occidentale* which is currently under threat mainly due to habitat degradation and water scarcity, risks that are further potentiated by its low geographic range and reduced genetic diversity. Although *A. occidentale* has been the target of an *ex situ* conservation program since 2007, there is still a considerable lack of baseline information on this species.

This study aimed to gather data on habitat use, population abundance and condition, growth and reproductive behaviour that could provide valuable guidelines to future restocking actions. Fish and habitat surveys were conducted in persistent pools that could act as summer refugia. Growth rates were determined for wild and captive populations and reproductive behaviour was studied in specimens bred in captivity.

Results showed that (1) macrophytes' coverage was positively associated and pool depth was negatively associated with fish relative abundance; (2) restocking actions led to a reinforcement of lower age classes in *A. occidentale* wild populations; (3) captive populations of the Alcabrichel and Safarujo rivers have higher growth rates than the corresponding wild ones; and (4) aquatic vegetation is the preferred substrate for spawning in this species.

Taken together, these results suggest that future restocking actions should be accompanied by habitat recovery, focusing on river bank vegetation, and conducted in persistent pools with high water volumes and abundant aquatic vegetation. It is also recommended that these actions are followed by annual surveys in order to assess the recovery of wild populations of *A. occidentale*.

Keywords: endangered cyprinids, *ex situ* conservation, habitat use, growth, reproductive behaviour

1. INTRODUCTION

1.1. Current status and threats to Iberian cyprinids

Freshwater fishes are amongst the most threatened organisms in the world (Duncan and Lockwood 2001, Maceda-Veiga 2012, Carrizo *et al.* 2013). Cyprinids are the most diverse family, show a worldwide distribution (Nelson 2006) and include some of the world's most threatened species (Duncan and Lockwood 2001). However, due to their lack of economic and fishing value (Doadrio 2001), there is few funding to the conservation of these species, namely through captive breeding (Sousa-Santos 2014a).

In the Iberian Peninsula, the cyprinid fauna includes a large proportion of endemic species (Doadrio *et al.* 2001, Cabral *et al.* 2005), which is mainly a consequence of the persistence of geographic barriers that strongly reduce the possibilities of colonization of this region by freshwater fishes (Robalo *et al.* 2006). Indeed, the complete elevation of the Pyrenees and the refill of the Mediterranean after the Messinian salinity crisis seem to have played a major role in preventing further contacts between Iberian and, respectively, Central European and African river systems (Levy *et al.* 2009).

Most Iberian cyprinids are highly threatened (Smith *et al.* 2006, Hermoso and Clavero 2011). Indeed, the proportion of threatened species in this region is amongst the highest recorded in the IUCN assessment of the conservation status of freshwater fishes in Europe (Freyhof and Brooks 2011). This situation is mainly due to several human-caused threats, including pollution, damming, water abstraction, and the spread of exotic species (Cabral *et al.* 2005). Additionally, Iberian cyprinids generally display low genetic diversity (Sousa-Santos *et al.* 2013) and small geographic distribution ranges (Cabral *et al.* 2005, Doadrio *et al.* 2001), which enhance their risk of imperilment. Currently, 26 out of the 38 species of Iberian cyprinids raise some level of conservation concern (Cabral *et al.* 2005, Doadrio *et al.* 2001).

1.2. The western ruivaco *Achondrostoma occidentale*

In Portugal, cyprinids and other freshwater and migrating fishes form the second most threatened group of vertebrates, with 67% of the ciprinid species classified as Vulnerable, Endangered or Critically Endangered, in the Portuguese Red Book of

Vertebrates (Cabral *et al.* 2005). Of the 21 native cyprinid species currently found in Portugal, 6 are “Critically Endangered” and 6 are “Endangered” (Cabral *et al.* 2005, Freyhof and Kottelat 2008, IUCN 2015).

The western ruivaco *Achondrostoma occidentale* (Robalo, Almada, Sousa-Santos, Moreira and Doadrio 2005) is one of the cyprinids endemic to Portugal listed as “Endangered” by the IUCN (Freyhof and Kottelat 2008).This small species reaches a mean adult size of 100 mm fork-length (Robalo *et al.* 2008) and shows a reduced distribution range, occurring only in three small independent basins in the western slope of Portugal: Alcabrichel, Sizandro and Safarujo (**Figure 1.1**) which face severe pollution, water abstraction, and habitat destruction (Robalo *et al.* 2008). The reproductive season extends from late April to early May (Robalo *et al.* 2008) and spawning seems to occur in aquatic plants, according to studies in captivity (Pereira 2007).

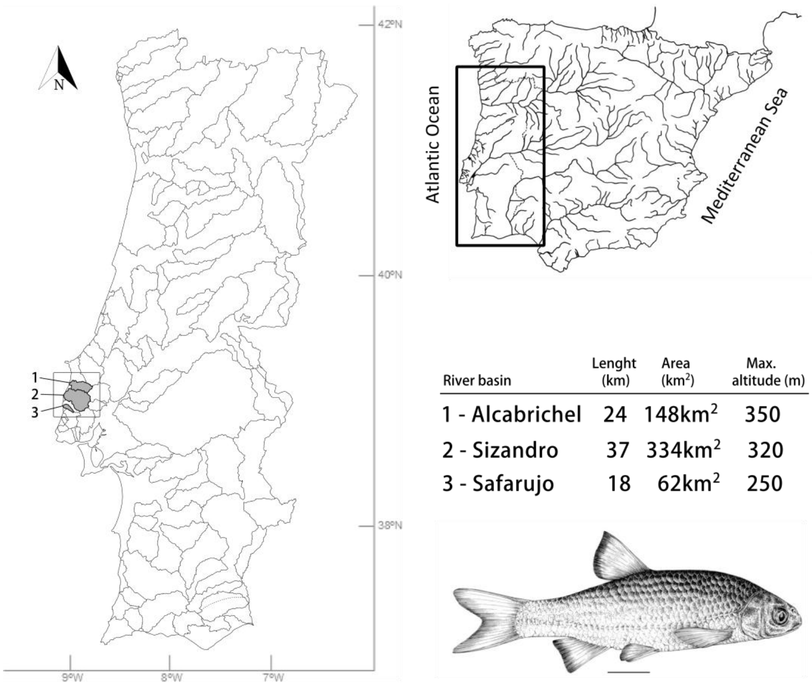


Figure 1.1. Location and general characteristics of the river basins inhabited by the western ruivaco *A. occidentale* [Illustration by Clara Almada].

Morphologically, *A. occidentale* resembles to the other two species of the *Achondrostoma* genus occurring in Portugal (*Achondrostoma arcasii* and *Achondrostoma oligolepis*), with orange coloration in the insertion of the pectoral, pelvic and anal fins (Robalo *et al.* 2005) - see **Figure 1.2**. Phylogenetically, *A. occidentale* is close to *A. arcasii* from the northwestern Iberian river basins (Robalo *et* *al.* 2006). A recent genetic survey revealed that *A. occidentale* populations are currently genetically depleted (Sousa-Santos *et al.* 2013) - see **Appendix I**.

Figure 1.2. *A. occidentale* live specimen. Photo credits: Jörg Freyhoff.

1.3. Captive breeding as a species conservation measure

After the description of *A. occidentale*, surveys conducted in 2005 and 2006 in the Alcabrichel, Sizandro and Safarujo rivers revealed that populations were small and fragmented (C. Sousa-Santos *unpublished data*). Moreover, a severe drought was still in course at the end of 2005 (García-Herrera *et al.* 2007), and long river stretches were already dry, increasing the risk of loss of individuals and local extinctions. In these circumstances, a stock of adult individuals from the Alcabrichel River was captured and maintained in a tank at the Vasco da Gama Aquarium as an extreme safeguard measure (Sousa-Santos *et al.* 2014). Sizandro wild specimens were also captured, but they were first used for a study on reproductive behaviour (Pereira 2007) and only afterwards stocked for captive breeding, at the Campelo Station (**Table 1.1**).

The spontaneous reproduction of this species in captivity (Gil *et al.* 2010) prompted the launching of an *ex situ* conservation program targeting *A. occidentale* and other five highly threatened cyprinids (see details in Sousa-Santos *et al.* 2014a). Similarly to other captive breeding programs (*e.g.* Bice *et al.* 2013, Hammer *et al.* 2013), the final goal of this program was to release captive bred populations in the streams where their founders were collected, after the requalification of at least some river stretches (Sousa-Santos *et al.* 2014). Indeed, captive breeding has been shown to be essential in preventing the extinction of several threatened species (*e.g.* Adamski *et al.* 2007, Rakes *et al.* 2013), by providing a high number of offspring *per* generation which can be used to restock wild populations with low effective population sizes (Champagnon *et al.* 2012).

Because avoiding artificial selection and domestication is crucial in *ex situ* conservation programs aiming to restock wild populations (Saura *et al.* 2008), a ‘‘naturalistic approach’’ of the reproduction was adopted for *A. occidentale*, aiming to breed and rear fish in conditions that would preserve the natural behavioural patterns of the species (Sousa-Santos *et al.* 2014a). This approach relied on: (1) naturally occurring spawning, without artificial stimulation or hormonal induction, (2) availability of refuge areas for fry and juveniles; (3) natural conditions of light and temperature, essential for gonadal maturation; and (4) minimal human intervention (Sousa-Santos *et al.* 2014a). To account for the eventual genetic diversity loss due to inbreeding and lineage sorting, captive breeding from the same initial stock was, in general, limited to three consecutive generations (one *per* year), and after the release of all the fish produced, new captive populations were founded from newly captured wild individuals (Sousa-Santos *et al.* 2014). Further details on the methods used and full descriptions of the *ex situ* conservation facilities can be found in Sousa-Santos *et al.* (2014a).

Since 2006, under the scope of the *ex situ* Conservation Program, seven captive stocks of *A. occidentale* were founded at the Vasco da Gama Aquarium and at the Campelo Station, and their offspring were used to restock their respective wild populations (Sousa-Santos *et al.* 2014a and *unpublished data*) - see **Table 1.1**.

Table 1.1. Year of foundation of the captive breeding populations of *A. occidentale* at the Vasco da Gama Aquarium (AVG) and at the Campelo Station (CS), and of the restocking actions in wild populations. Note: Restocking of the Safarujo population in 2015 occurred after the collection of the data presented in this thesis.

River basin	*Ex situ* facilities	Introduction in captivity	Number of spawners	Restocking action	Number of individuals released
Alcabrichel	AVG	2006	16	2011	400
Alcabrichel	CS	2009	45	2011	400
Alcabrichel	CS	2011	60	2013	1190
Alcabrichel	AVG	2011	60	2013	446
Alcabrichel	CS	2013	60	2015	2482
Sizandro	CS	2009	19	2013	1309
Safarujo	AVG	2012	12	2015	350

Overall, between March 2011 and April 2015, seven restocking actions of the wild populations were conducted, comprising the release of 6577 fish reared in captivity (**Table 1.1**): five restocking actions at the Alcabrichel River (N=4918 individuals), one at the Sizandro River (N=1309 individuals) and one at the Safarujo River (N=350) (Sousa-Santos *et al.* 2014 and *unpublished data*). In all cases, fish were released in upstream stretches, in areas with preserved habitats and good water quality (see **Figure 3.1** in **Material and Methods** for site locations), after being marked by cutting off the dorsal fin, for easy identification of recaptured individuals in future surveys. Fin cut off has been shown to have minor effects on fish, with scars persisting quite visible over time (Sousa-Santos *et al.* 2014a). Nevertheless, to avoid injuring small fish, individuals smaller than 50 mm fork-length were not marked. As it is essential that *ex situ* conservation programs are supported by habitat restoration actions, some efforts were also made concerning water quality improvement and habitat rehabilitation, especially at the Alcabrichel River, where natural engineering techniques were used to recover spawning habitats and prevent river bank erosion (Sousa-Santos *et al.* 2014a).

1.4. Integrating ecological and ethological data into restocking programs

Besides captive breeding and habitat restoration, the adoption of effective measures towards the conservation of endangered species generally benefits from multidisciplinary data on the target species, namely on habitat influence (Granado-Lorencio 1992), population prioritization, dynamics and distribution (Magalhães *et al.* 2002a, Magalhães *et al.* 2003, Clarkson *et al.* 2012), genetics and evolutionary history (Robalo *et al.* 2007, Karaiskou *et al.* 2011, Kitanishi *et al.* 2013), behaviour (Sutherland 1998, Robalo *et al.* 2007, Ozer and Ashley 2013) and habitat use (Santos and Ferreira 2008, Martelo *et al.* 2014). Given the characteristics of the rivers inhabited by *A. occidentale*, with a high level of pollution and flow intermittence (ARHTejo 2011), and the fragmentation revealed in these species populations (Sousa-Santos *et al.* 2014a), the later approaches towards the conservation of endangered species can assume a vital role in this specific case.

Rivers in southern Iberian Peninsula are generally intermittent, showing seasonal floods and droughts of variable intensity, in association with fluctuations in rainfall (Granado-Lorencio 1992, Clavero *et al.* 2004). Drying is likely to be particularly significant for fish, as it results in major habitat contraction and loss of connectivity throughout the stream network (Magalhães *et al.* 2002b). This particular context makes it essential to understand which habitats may act as refugia and favour the persistence of *A. occidentale* along the river courses during the dry season, as those should be considered priority areas for protection and restoration and may configure potential locations for restocking actions.

Ethological studies can also have an essential contribute to the conservation of *A. occidentale*, as it has already been demonstrated that data on the spawning behaviour should be taken into account in the context of fish conservation, and particularly of captive breeding and restocking (Rakes *et al.* 1999, Carvalho 2003, Carvalho *et al.* 2003, Pereira 2007, Goren 2009, Sousa-Santos *et al.* 2014). Although all cyprinids are oviparous with external fertilization, there is high variability in their behavioural patterns (Pereira 2007). Until now, studies on the spawning behaviour of Portuguese native cyprinids were only conducted for *A. oligolepis* and *A. occidentale* (Pereira 2007), *Iberochondrostoma lusitanicum* (Carvalho *et al.* 2003), *Pseudochondrostoma polylepis* (Robalo *et al.* 2003), *Squalius pyrenaicus* (Sousa-Santos *et al.* 2014b) and *Squalius alburnoides* (Sousa-Santos *et al.* 2006).

One example of how these studies may have implications for species conservation is the captive breeding of *S. pyrenaicus* at the Vasco da Gama Aquarium: after two years without spawning, this species finally reproduced when the tank was supplemented with coarse substratum (Sousa-Santos *et al.* 2014b). The existence of preferred substrates is common among cyprinids (*e.g.* Johnston 1999) and, thus, preserving those spawning grounds may be crucial for species recruitment and ultimately for species survival. Regarding *A. occidentale*, Pereira (2007) found some evidence of preference for egg laying on plants when studying a group of breeding fish in a small aquarium. However, it is important to test this preference at a larger scale and with a higher number of individuals, so that captive breeding may be optimized and substrate preference for spawning may be taken into account when choosing sites for restocking of wild populations of *A. occidentale*.

Life history patterns should also be taken into account for effective conservation measures to protect threatened species (Aparicio and Sostoa 1998). In particular, knowledge of variation in traits such as age and sex composition, growth in length and weight, length-weight relationships, somatic condition, spawning time and age of maturity and fecundity is particularly important. (Andreu-Soler *et al.* 2006, Miranda *et al.* 2006, Sarkar *et al.* 2013). For instance, analyses of growth and condition of *A. occidentale* along habitat gradients will provide significant insights on which sites are more favourable to the growth of juveniles and, consequently, potentially configure releasing sites during restocking actions.

1.5. Objectives

This work follows a multidisciplinary approach to gather some baseline but essential information on habitat use, growth and reproductive behaviour of wild and captive populations of *A. occidentale*, which may contribute to improve the efficiency of future restocking of their natural populations. Specific aims were to (i) quantify relative abundances, age and condition of fish in persistent pools that can act as summer refugia for this species, and habitat factors that may influence these; (ii) analyze the growth patterns of captive populations and compare them with those of wild populations; and (iii) describe the reproductive behaviour of *A. occidentale* in captivity and assess substrate preferences for spawning.

Concealing all the data gathered, this work will contribute with key aspects on the ecology of *A. occidentale* and on the differences in population traits between wild and captive populations, which might be useful to achieve more efficient reinforcements under the scope of the ongoing *ex situ* conservation program and, consequently, to the conservation of the species.

2. STUDY AREA

The Alcabrichel, Sizandro and Safarujo rivers, ranging from 18 to 37 km in length (**Figure 1.1**), are shaped by highly seasonal flows, showing reduced or no flow in the summer and large floods in the winter. During the summer, the streams are often reduced to a series of disconnected pools, which represent the only chance of fish to persist by acting as refugia and as sources of recruits or colonists for the entire system, after flow resumption at the end of the dry season (Magalhães *et al.* 2002, Pires *et al.* 2014).

Moreover, these rivers face strong habitat destruction and pollution, especially due to sewage from pig farms, distilleries, and agricultural and urban discharges (Robalo *et al.* 2005, Robalo *et al.* 2008). Indeed, the Alcabrichel River has been classified as highly degraded, based on the results obtained for the biotic index based on macroinvertebrates IBMWP (Teixeira *et al.* 2008) and the Safarujo River is known to dry out almost completely in years of severe droughts. Indeed, the population of *A. occidentale* of the Safarujo River was thought to be extinct until 2011 (Sousa-Santos *personal communication*).

There is also a high abundance of *Arundo donax* along the margins of these streams, preventing native vegetation from growing and stabilizing the river banks (**Figure 2.1**). Riparian vegetation is sparse and constantly under removal (ARHTejo 2011), and includes the narrow-leaved ash *Fraxinus angustifolia*, the white poplar *Populus alba* and the willow *Salix salvifolia*.

Besides *A. occidentale*, fish assemblages include the European Eel *Anguilla anguilla* and the loach *Cobitis paludica.* The presence of exotic fish species was not reported so far. The invasive red swamp crayfish *Procambarus clarkii* is also abundant in the studied rivers.

Figure 2.1. *Arundo donax* plantations along the Alcabrichel River.

3. MATERIAL AND METHODS

3.1. Survey of wild populations in summer refugia

Surveys of wild populations of *A. occidentale* were conducted at the end of the dry season, immediately before the rains, when fish remain gathered in persistent pools, which act as summer refugia and sources of colonization for the entire rivers after flow resumption. Specifically, surveys for fish and habitat conditions in persistent pools, were conducted between 11th September and 8th October 2014 in the Alcabrichel, Sizandro and Safarujo rivers.

The upper and medium courses of the each river were prospected by car in order to map (WGS84 datum coordinate system) all the pools persisting in the channel. Lower courses of the rivers were not surveyed because they show high habitat degradation and poor water quality, and thus are not suitable for future restocking actions. The prospected stretches in the upper and medium courses of the Alcabrichel, Sizandro and Sarafujo Rivers were approximately 13.75, 15.60 and 11.24 km long, respectivelly. Overall, 14 pools persisted in the Alcabrichel (6), Sizandro (3) and Safarujo (5) rivers - **Figure 3.1**.

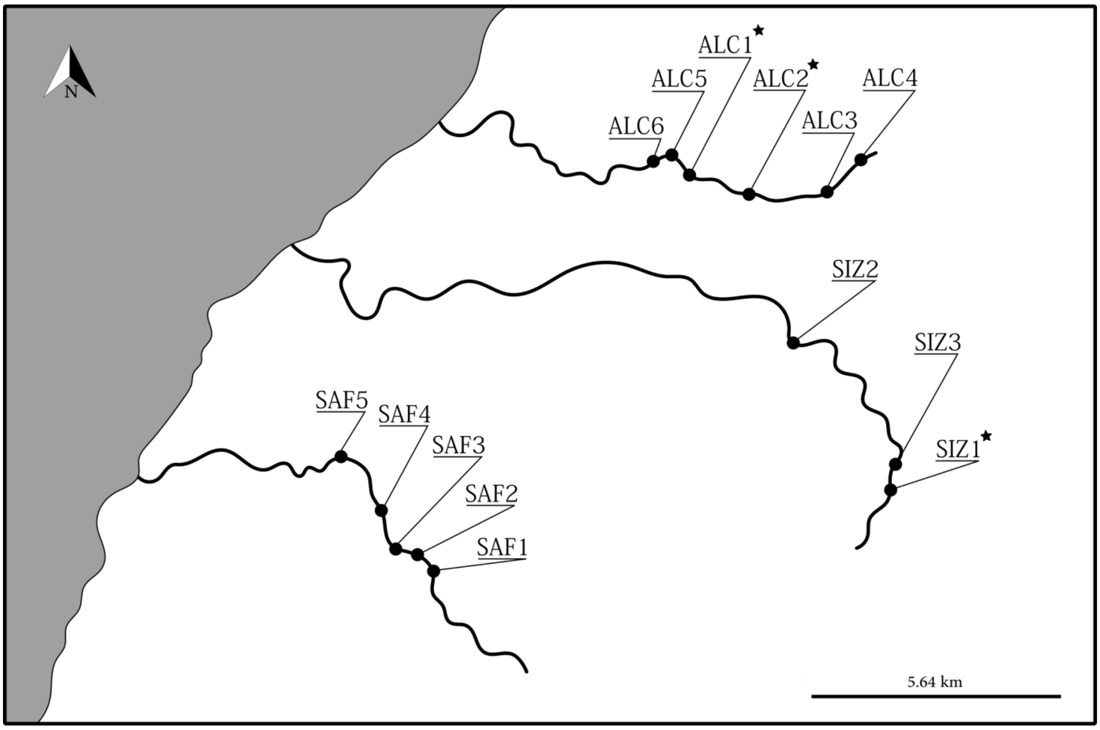


Figure 3.1. Persistent pools in the middle and upper courses of the Alcabrichel (ALC1 to ALC6), Sizandro (SIZ1 to SIZ3) and Sarafujo (SAF1 to SAF5) rivers surveyed for fish and habitat conditions.
* pools subjected to fish restocking actions prior to the collection of the data presented herein.

Fish sampling was performed with a SAMUS725G electrofishing portable device. Each pool was slowly prospected in a zigzag manner from the downriver to the upriver edge. In order to get an estimate of the fish relative abundance, time of fishing was roughly proportional to pool size (**Appendix II**). All captured fish were maintained in permanently aerated buckets, measured (fork length, nearest millimetre) and weighted (total body weight, nearest gram), and three scales were taken from individuals with over 40 mm length, from the third scale row below the dorsal fin (**Appendix III**). The scales were stored in field sheets with scotch tape for transportation and posterior age and growth analyses (see section 3.3). The dorsal fin of each fish was inspected for the presence of a horizontal scar (mark used to identify individuals bred in captivity - see section 1.3). Finally, all individuals were returned to the river.

The habitat at each pool was described using 15 variables: maximum length (in meters), maximum width (in centimetres), maximum depth (in centimetres), area (in m^2), volume (in m^3), macrophytes' coverage, instream coverage and detritus abundance (0: 0%, 1: <30%, 2: 30-60%, 3: >60%), dominant substrate [(1: silt (4-64) µm, 2: sand (64 µm-2 mm), 3: gravel (2-64 mm), 4: cobbles (64-256 mm), 5: boulders (>256 mm)], substrate heterogeneity (number of substrate categories), water temperature (in ºC), ammonia (in mg/L), nitrites (in mg/L), nitrates (in mg/L) and pH (**Table 3.1**). Photos of some of the procedures used to determine habitat variables can be found in **Appendix IV**.

Table 3.1. Variables used to characterize the habitat in the summer refugia. The number of pools sampled (n), and the abbreviation, scale and unit of each variable are also presented.

Variable	Abbreviation	n	Scale	Unit
Maximum length	-	14	-	m
Maximum width	-	14	-	m
Maximum depth	DEPTH	14	-	m
Area	AREA	14	-	m^2
Volume	VOL	14	-	m^3
Macrophytes' coverage	PLANT	14	0-3	-
Instream coverage	SHADE	14	0-3	-
Detritus abundance	DET	14	0-3	
Dominant substrate	DSUB	14	1-5	-
Substrate heterogeneity	SUBH	14	1-5	-
Water temperature	TEMP	14		ºC
Amonia	-	13	-	mg/L
Nitrites	NO2	14	-	mg/L
Nitrates	NO3	14	-	mg/L
pH	-	12	1-14	-

The area and volume of the pools were calculated by approximation to an ellipsoid, as follows:

$$Area = 4\pi\left(\frac{a^p b^p + a^p c^p + b^p c^p}{3}\right)^{1/p}$$

$$Volume = \frac{4}{3}\,\pi abc$$

where *a*, *b* and *c* represent, respectively, the maximum length, width and depth of each pool, and *p* (relative error=1.61) the maximum relative error associated with the approximation to an ellipsoid (Xu *et al.* 2009).

3.2. Survey of captive populations

Captive populations of *A. occidentale* maintained at the Vasco da Gama Aquarium (AVG) and at the Campelo Station (CS) were surveyed between March and April 2015. In order to minimize the disturbance of these populations, only a small number of individuals were sampled for fork-length and scale analyses (**Table 3.2**).

After lowering the water level, the tanks were prospected with hand nets and the captured fish were maintained in permanently aerated buckets. All fish with at least 40 mm were measured (fork-length, nearest millimetre) and three scales from the third scale row below the dorsal fin were collected.

Table 3.2. Foundation year of captive populations analysed in this thesis, total number of individuals (N) in each population in the Spring of 2015, and number of individuals sampled (n).

Source population	Facility	Foundation	N	n
Alcabrichel	CS	2013	2482	102
Sizandro	CS	2013	760	18
Safarujo	AVG	2012	250	51

3.3. Age and growth

Age was determined through scale reading, by counting the number of annuli, corresponding to the number of years (Chilton and Beamish 1982). Scales were mounted on microscope slides with a water drop and the annuli were interpreted. Scale and annuli radiuses were measured in the right side of the anterior field of the scale, which generally showed low pigmentation and clear evidence of annuli (**Figure 3.2**).

Measurements were performed using *Motic Images Plus* software (Motic China Group, version 2.0).

Fork-length at age *i* was back-calculated from linear regression equations, following the body-proportional hypothesis described by Rasmussen *et al.* (1996). Equations used in calculations are presented in **Appendix V**.

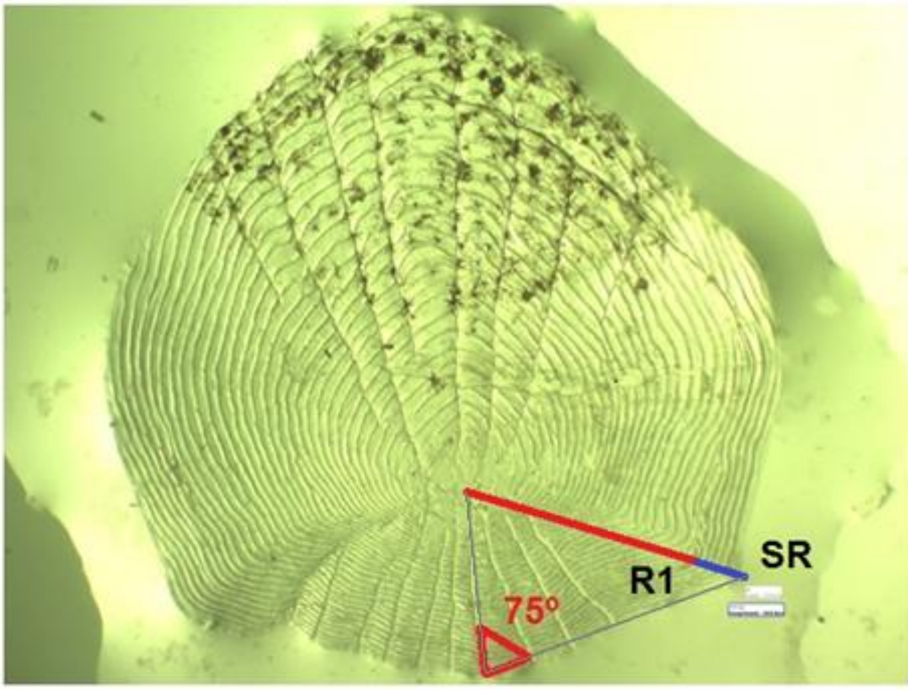


Figure 3.2. Measuring of the radius to the first annuli (R1, red line) and total scale radius (SR, red line plus blue line) radiuses in a scale taken from a 1+ specimen caught during the summer refugia survey. Radiuses were measured to the right anterior edge of the scale. The centre and right anterior edge of each scale were determined by establishing a 75º angle between two auxiliary lines (in grey).

3.4. Reproductive behaviour

Observations of the spawning behaviour of *A. occidentale* were conducted with a captive population from the Safarujo River, founded with 12 wild adults in 2013, at the Vasco da Gama Aquarium. In 2015, this captive population consisted of 369 individuals, 44 of which were adults (>70 mm fork-length) and 325 were juveniles (<70 mm fork-length). As reproductive behaviour is not performed by juveniles, only adults were considered for this study. Mature females were identified by their swollen abdomen, whereas mature males were recognized by their fusiform body, presence of nuptial tubercles in the head and conspicuous behaviours performed towards females.

The experimental tank (designated as T9 in **Figure 3.3**) had a capacity of 3120 L (500x195x32 cm), continuous water supply and regulation, and two permanent air sources to maintain regular levels of dissolved O_2. Because the captive breeding of this species followed a naturalistic approach (described in detail in Sousa-Santos *et al.* 2014a), bricks and a cage with a small mesh diameter were placed in the tank in order to provide shelter for juveniles and larvae, respectively. A wood pier was also present to provide shading to about one third of the tank, resembling the instream coverage provided by the riparian gallery in wild habitats. The tank was also supplied with pots containing aquatic plants with abundant submerged roots (*Cyperus alternifolius*, *Echinodorus* sp., *Egeria densa*, *Elodea* sp. and *Typha latifolia*) - see **Figure 3.4**.

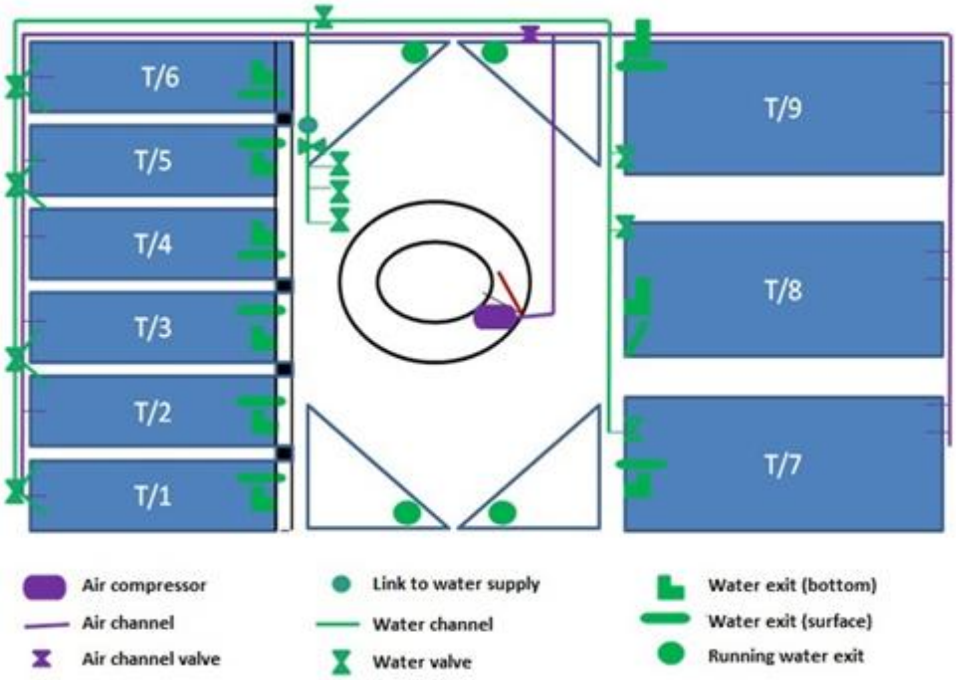


Figure 3.3. Schematic sketch of the *ex situ* conservation facility at the Vasco da Gama Aquarium, The observations on the spawning behaviour of *A. occidentale* were conducted in tank T9.

Fish behaviour was recorded with an underwater camera (Nikon Coolpix AW100) placed in fixed positions to minimize disturbance and stress. The quantitative analysis of the reproductive behaviour data was preceded by a period of informal observations (from 11th December 2014 to 25th March 2015), aiming at defining the typical behaviour of the species off the reproductive season, and by a period of preliminary observations (from April 2nd to April 30th), aiming at defining the behavioural patterns associated with reproductive behaviour and determining the ethogram of the species, as advised by Martin and Bateson (1986). The period of preliminary observations began when males started performing typical reproductive behaviours, namely long runs and female chasing (described in detail by Pereira 2007).

After the description of the ethogram of the species, observations of the reproductive behaviour were carried out (from 5th May 2015 to 24th May 2015) during three periods of the day to account for eventual variations of fish activity throughout the day: i) 8h30 to 11h30, ii) 11h30 to 14h30, and iii) 15h00 to 18h00. As feeding took place between 14h30 and 15h00 thrice a week, no records were taken during that time period to avoid bias in behaviour due to feeding.

Substrate preferences for spawning were assessed using an experimental design similar to that described by Pereira (2007). Within each of the time periods described above, recording sessions with 20 minutes of duration were conducted at three distinct substrate sites (each site was sampled once or twice): plants, wool spawning mops (see **Appendix VI**) and gravel. The aquatic camera used to record observations was placed at 27 cm depth when sampling the sites with plants and wool mops, and at 22 cm depth when sampling the gravel site. In each recording session, the absolute frequency of the behaviours described in the ethogram was quantified.

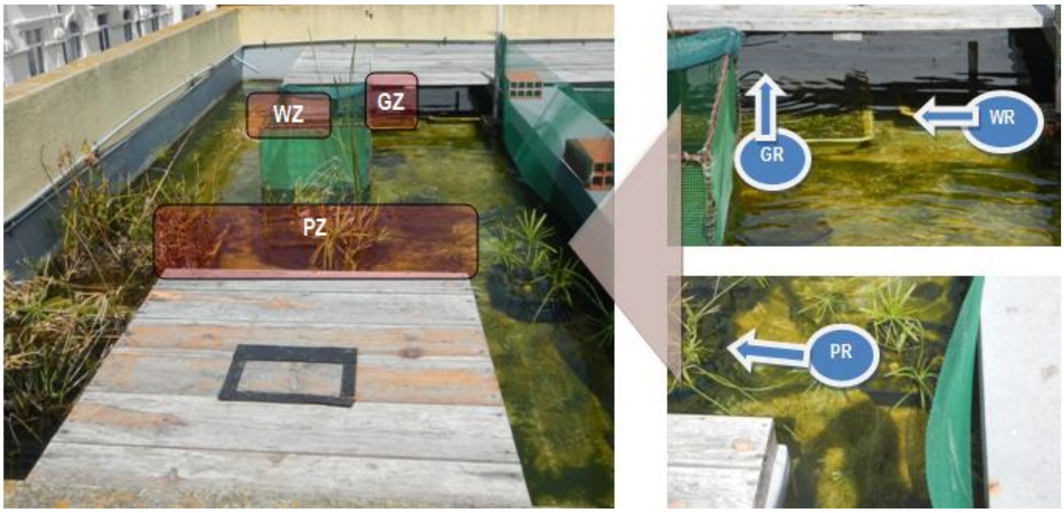


Figure 3.4. Experimental tank used for the ethological study of the reproduction of the captive population of *A. occidentale* from Sajarujo, at the Vasco da Gama Aquarium. Left: sampling sites occupied by plants (PZ), wool spawning mops (WZ) and gravel (GZ); Right: location of the camera to record reproductive behaviours occurring in wool spawning mops (WR), gravel (GR) and plants (PR). Arrows indicate the direction of recording.

To assess the eventual effect of temperature and light in the onset of reproductive behaviours of *A. occidentale*, one data logger (Hobo® Pendant UA-002) was installed in the experimental tank on 9th April 2015. Data concerning water temperature (in ºC) and light quantity (in lux) were automatically recorded every four hours (7:00, 11:00, 15:00, 19:00, 23:00, 3:00) until the end of the study (24th May 2015).

3.5. Data analyses

Data analyses focused on quantifying variation in population traits between wild and captive populations and on assessing patterns on the reproductive behaviour of *A. occidentale*. Prior to analyses, data were assessed for normality and homogeneity, and non-parametric statistics were used when these assumptions were not met. Significance of statistical testing was assessed at $p<0.05$ and analyses were conducted using the *R* software (The R Foundation for Statistical Computing, version 3.0.3) and *STATISTICA* software (Dell Inc., version 12). Statistical procedures are detailed in sections 3.5.1 and 3.5.2.

3.5.1. Relative abundance of fish in summer refugia

The number of fish caught in each pool was used to index its relative abundance, and further related to habitat gradients prevailing in the study area. Principal Components Analysis (PCA) was used to describe the main gradients of variation in habitat conditions. To avoid bias in the ordination, all habitat variables that were not measured in all persistent pools (n=14, see **Table 3.1**), i.e. ammonia and pH, were excluded from the analyses. Likewise, maximum length and width were also excluded, because they were considered for both pool area and volume calculations, To minimize collinearity problems, whenever pairs of habitat variables were strongly correlated (Pearson's r, $|r| \geq 0.80$; **Appendix VII**), only the variable most correlated with relative abundance from each pair was retained for analyses. Principal components (PC) axes were then used as explanatory variables in linear regression analyses relating fish relative abundance to habitat gradients.

3.5.2. Fork-length distributions and condition factor in wild populations

Fork-length distributions were built for each population by pooling data from persistent pools. Size class amplitude used in histograms was of 2 mm.

Condition factor for each individual was determined using the scaled mass index (SMI), following the procedure described by Peig and Green (2009). Specifically, the SMI was calculated using the following formula adapted from Peig and Green (2009),

$$SMI = W \times \left(\frac{FL_0}{FLi}\right)^{b_{SMI}}$$

where *W* is the measured weight, FL_0 the observed mean fork-length in each river, FL_i the fork-length measured for each individual and b_{SMI} the slope of the linear regression equation of weight and fork-length log-transformed data.

The Kruskal-Wallis test was used to test for variation in fork-length and in the SMI among populations, and the *post hoc* Dunn test was performed whenever significant differences were found. Fish length at each pool (mean, minimum and maximum) was related to habitat gradients extracted from PC axes using linear regression analyses.

3.5.3. Age and growth

The back-calculated lengths at age *i* were compared among wild and captive populations by using the one-way ANOVA test, followed by the Tukey *post hoc* test whenever significant differences were found. Comparisons between wild and captive populations were also conducted using either the t-test (for the Alcabrichel and Safarujo populations) or the non-parametric Mann-Whitney test (for the Sizandro population).

3.5.4. Factors shaping reproductive behaviour

To test whether the frequency of behaviours across substrates was independent of the time period during which the observations were recorded, a chi-square of independence was performed. A chi-square test of homogeneity was also used to test for variation of the frequency of behaviours amongst substrates. In both cases, behaviours with frequencies below 5 were excluded from analysis to avoid lowering inference reliability (Roscoe and Byars 1971).

The pooled absolute frequencies of each behaviour *per* day, taken as an indicator of the fish reproductive activity, were plotted against the average daily temperature and light incidence to assess eventual associations between fish activity and temperature/light incidence.

4. RESULTS

4.1. Habitat variability in summer refugia

Persistent pools ranged between 45.39 m^2 (in Safarujo) and 268.49 m^2 (in Sizandro) in area and between 4.83 m^3 (in Safarujo) and 63.38 m^3 (in Alcabrichel) in volume (**Table 4.1**). In average, pools in the Alcabrichel River showed the highest water volume and were the longest and deepest, while pools in the Sizandro River were the largest in area and width, the shallowest and the ones with the highest instream and macrophytes' coverages. The pools in the Safarujo River were, in average, the smallest in area, volume, length and width and the ones with the lowest macrophytes' coverage. Substrate was dominated by silt in Alcabrichel and by sand in the Sizandro and Safarujo rivers. The average amount of nitrites and nitrates was higher in the Alcabrichel and Sizandro rivers, whilst ammonia was higher in the Sarafujo (0.12±0.16); average pH ranged between 7.36 in Alcabrichel and 8.00 in Sizandro.

Table 4.1. Habitat conditions in the summer refugia in Alcabrichel, Sizandro and Safarujo rivers. Values are the mean±SD and the range (minimum-maximum) for each variable.

River basin	Alcabrichel (n=6)	Sizandro (n=3)	Safarujo (n=5)
Maximum length (m)	19.47±9.10 (6.40-31.43)	16.57±10.56 (4.65-24.76)	12.01±3.93 (8.80-18.84)
Maximum width (m)	4.33±1.09 (2.55-5.80)	7.15±3.17 (4.21-10.50)	4.12±0.99 (2.68-5.46)
Maximum depth (cm)	62.17±26.79 (30-100)	44.67±16.74 (35-64)	58.80±29.69 (33-109)
Area (m^2)	136.69±72.88 (62.82-224.53)	161.37±25.36 (78.40-268.49)	81.54±34.74 (45.39-139.36)
Volume (m^3)	27.44±20.61 (8.11-63.38)	26.82±25.36 (8.95-55.84)	17.62±16.68 (4.83-46.77)
Macrophytes' coverage	1.33±0.52 (1.00-3.00)	2.33±0.58 (1.00-3.00)	0.80±0.45 (0-3.00)
Instream coverage	2.00±1.10 (1.00-3.00)	2.33±1.15 (1.00-3.00)	2.00±1.00 (1.00-3.00)
Dominant substrate	1.33±0.52 (1.00-2.00)	2.00±1.00 (1.00-3.00)	2.00±1.22 (1.00-4.00)
Ammonia (mg/L)	0.05±0.05 (0-0.10)	0.05±0.07 (0-0.10)	0.12±0.16 (0-0.40)
Nitrites (mg/L)	0.46±0.33 (0.05-0.80)	0.50±0.36 (0.10-0.80)	0.05±0.03 (0.03-0.10)
Nitrates (mg/L)	18.33±18.35 (0-40)	13.33±11.55 (0-20)	0.20±0.45 (0-1.00)
pH	7.36±0.54 (6.4-7.6)	8.00	7.72±0.23 (7.4-8.0)

Correlation analyses showed that volume and nitrites were strongly correlated with area and nitrates ($r>0.80$), respectively, but less correlated with fish relative abundance than their respective pair (see **Appendix VII**). In these circunstamces, volume and area were not further considered in the ordination.

The first three componens of the PCA explained 65.5% of the variation in the habitat data (**Table 4.2**). The first component (PC1, 25.2% of total variance) reflected a gradient of increasing water temperature, macrophytes' coverage and nitrates, and decreasing depth. PC2 explained 21.3% of total variance and was associated with increasing detritus abundance and decreasing substrate size. Finally, PC3 explained 19.0% of total variance and was positively associated with pool area and substrate heterogeneity.

Table 4.2. Percentage of variance explained by the first three PC axes and habitat variables most associated with each axis. Only variables with factor loadings higher than |0.60| are presented.

Habitat variable	PC1	PC2	PC3
Maximum depth	-0.64	-	-
Area	-	-	0.84
Macrophytes' coverage	0.63	-	-
Detritus abundance	-	0.90	-
Dominant substrate	-	-0.87	-
Substrate heterogeneity	-	-	0.79
Water temperature	0.78	-	-
Nitrates	0.64	-	-
% of explained variance	25.2	21.3	19.0

The PC1 contrasted persistent pools SIZ3 and ALC2 having lower depths and higher water temperatures, macrophytes' coverage and nitrates with pools SAF1, SAF4 and ALC 4 e ALC5 (**Figure 4.2**). These later two pools showed the highest abundance of detritus and smaller substrates, contrasting with SAF1 and SIZ2 along PC2 (**Figure 4.2**). ALC5 and SIZ3 showed larger areas and higher substrate heterogeneity, in opposition to SAF2 and SAF3 along PC3 (**Figure 4.2**).

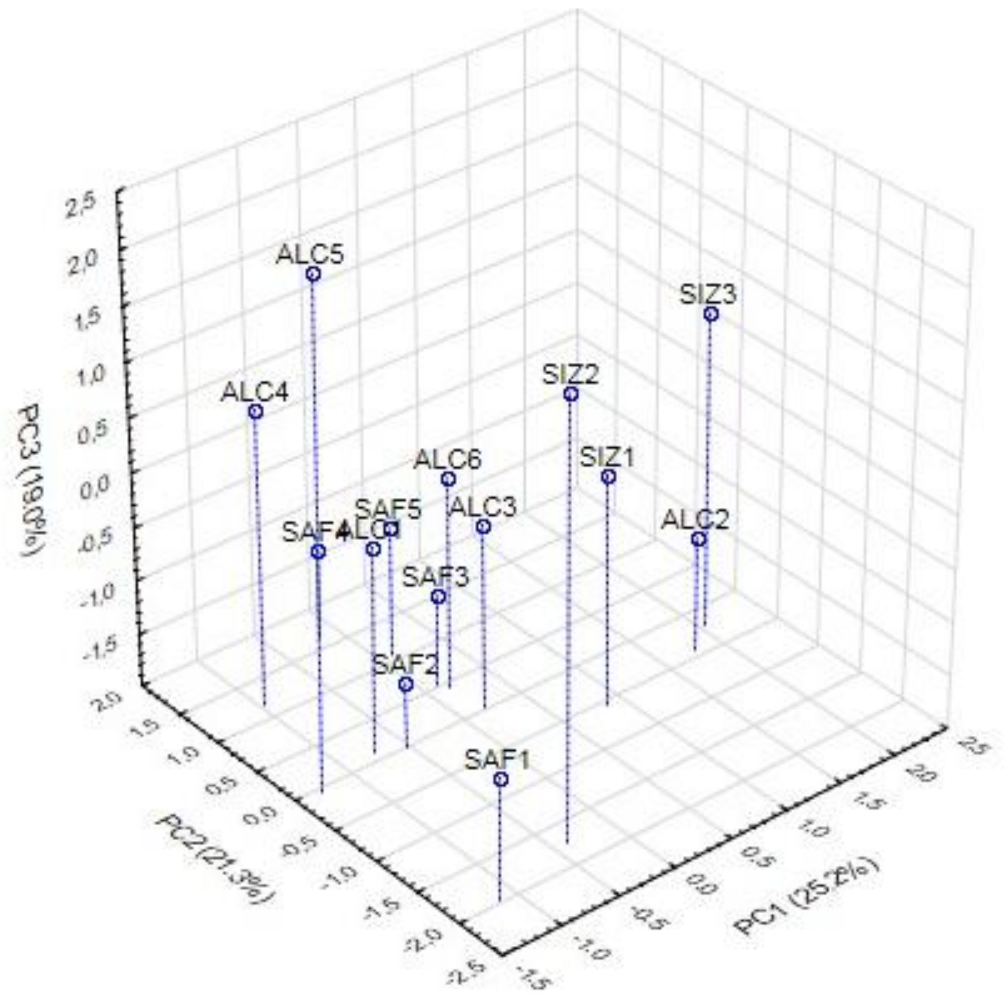


Figure 4.2. Ordination diagram of PCA of habitat conditions in summer refugia in the Alcabrichel, Sizandro and Safarujo rivers. See **Figure 3.1** for codes and locations of pools in **Material and Methods**.

4.2. Fish relative abundance in summer refugia

A total of 1005 individuals were caught in the 14 persistent pools found in the Alcabrichel, Sizandro and Safarujo rivers (**Figure 4.3**). The number of fish *per* pool ranged from 9 individuals at SAF3 to 271 at SIZ3), with the average number of individuals *per* pool ranging between 27.0 in Safarujo and 164.7 in Sizandro (**Table 4.3**). The highest fish relative abundance values were detected at one of the restocking sites of the Alcabrichel River and dowstream from the restocking site for the Sizandro River (Figure 4.3).

Only 4 individuals had a clear horizontal scar in the dorsal fin, indicative of being from a captive facility: three at SIZ2 (fork-length ranging from 78 mm to 106 mm) and one at ALC6 (102 mm fork-length).

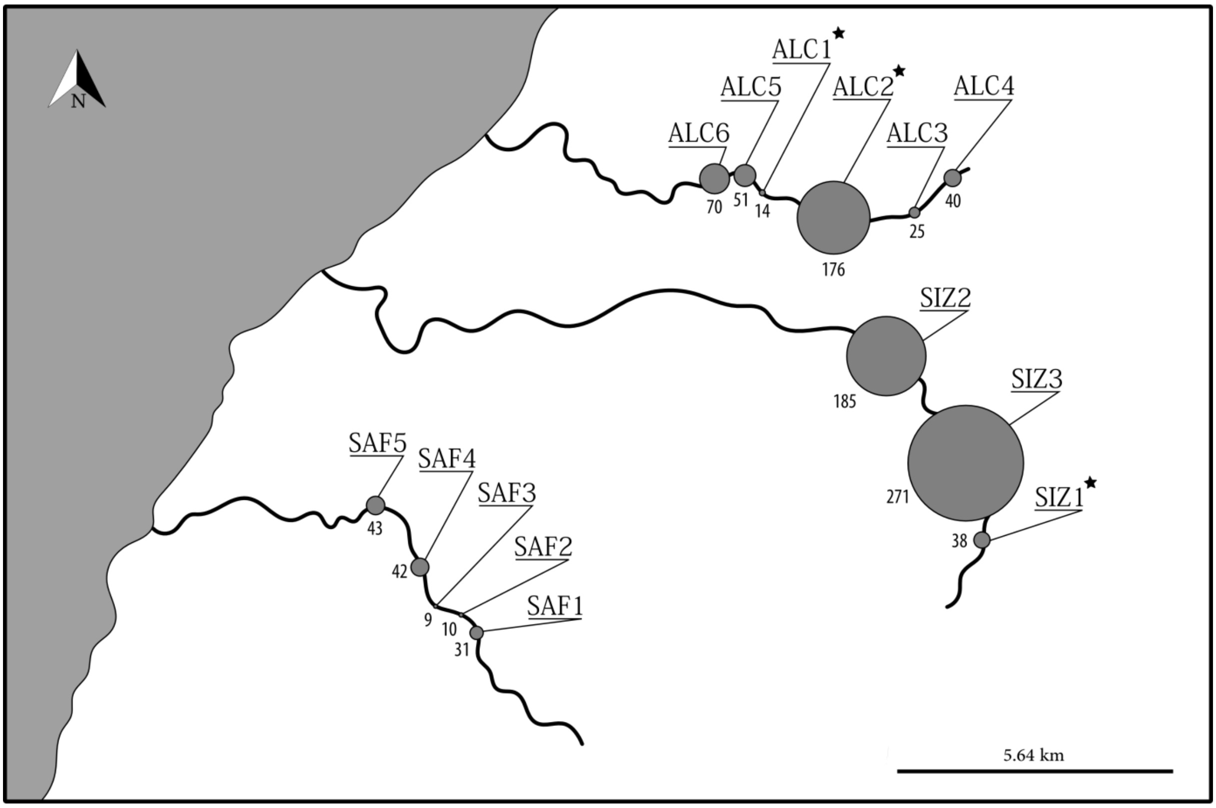


Figure 4.3. Relative abundance of *A. occidentale* in persistent pools in the middle and upper courses of the Alcabrichel (ALC1 to ALC6), Sizandro (SIZ1 to SIZ3) and Sarafujo (SAF1 to SAF5) rivers. Grey circles are proportional to the number of individuals caught in each pool. *pools where fish were released during restocking actions conducted prior to sampling.

Table 4.3. Number and fork length (FL, in mmm) of *A. occidentale* individuals caught *per* persistent pool in the Alcabrichel, Sizandro and Safarujo rivers. Mean±SD and range (minimum-maximum) values are presented for each variable.

River basin	Total No. individuals	No. individuals/pool mean±SD (min-max)	FL/pool mean±SD (min-max)
Alcabrichel	376	62.7±58.9 (14-176)	45.5±21.0 (18-110)
Sizandro	494	164.7±117.8 (38-271)	54.1±19.5 (19-130)
Safarujo	135	27.0±16.7 (9-43)	55.5±20.5 (23-107)

Fish relative abundance was significantly related to PC1 (R^2=0.45, F=9.809, p<0.009), but showed no association with PC2 (R^2=0.10, F=1.317, p<0.274) neither with PC3 (R^2=0.22, F=3.461, p<0.088).

4.3. Length of fish in wild populations

Individuals caught in persistent pools ranged in fork length from 18 mm at Alcabrichel (ALC2) to 130 mm at Sizandro (SIZ1), with an average size *per* population ranging between 45.5 mm in Alcabrichel and 55.5 mm in Safarujo (**Table 4.3**). SAF2 showed the lowest variability in fork-length (SD=3.4), ranging from 30 to 41 mm, while ALC1 and ALC4 had the highest ranges (SD=22.8). The highest mean was attained at ALC1 (63.3 mm), followed by SAF3 (59.8 mm), ALC5 (55.7 mm) and ALC6 (55.0 mm) - see **Table 4.4**.

Table 4.4. Mean fork-length (FL) ± standard deviation (SD) and range (minimum-maximum) values for individuals sampled in each summer pool; n=sample size.

Pool	Mean FL ± SD	Range (min-max)	N
ALC1	63.3 ± 22.8	28-110	14
ALC2	32.4 ± 11.6	18-87	176
ALC3	43.4 ± 20.8	25-89	25
ALC4	47.4 ± 22.8	23-90	40
ALC5	55.7 ± 19.6	23-106	51
ALC6	55.0 ± 19.3	28-90	69
SIZ1	54.1 ± 19.6	28-130	38
SIZ2	53.6 ± 18.9	19-107	185
SIZ3	48.2 ± 11.4	26-88	271
SAF1	54.4 ± 19.8	23-95	31
SAF2	34.4 ± 3.4	30-41	10
SAF3	59.8 ± 21.8	36-105	9
SAF4	54.9 ± 19.4	30-107	42
SAF5	54.7 ± 19.2	43-101	43

The mean and minimum fork-length of fish were not associated with any of the PC axes ($0.00<R^2<0.10$, $0.543<F<2.495$, $0.475<p<0.140$), but maximum fork-length showed a negative association with PC2 ($R^2=0.31$, $F=5.366$, $p<0.039$).

Mean fork-length obtained for the Alcabrichel and Sizandro populations showed the highest values in two persistent pools that were targets of restocking actions: ALC1 (restocked twice, in 2011 and 2013, SMI=63.3 ± 22.8) and SIZ1 (restocked once, in 2013, SMI=54.1 ± 19.6), respectively.

Overall, the populations from Alcabrichel and Sizandro presented bimodal length distributions, with modes in the 20-40 and 60-80 mm in the Alcabrichel and in the 40-60 and 60-80 mm in the Sizandro (**Figure 4.4**). Contrastingly, the distribution obtained for the Safarujo population was approximately normal, with the mode in the 40-60 mm class (**Figure 4.4**).

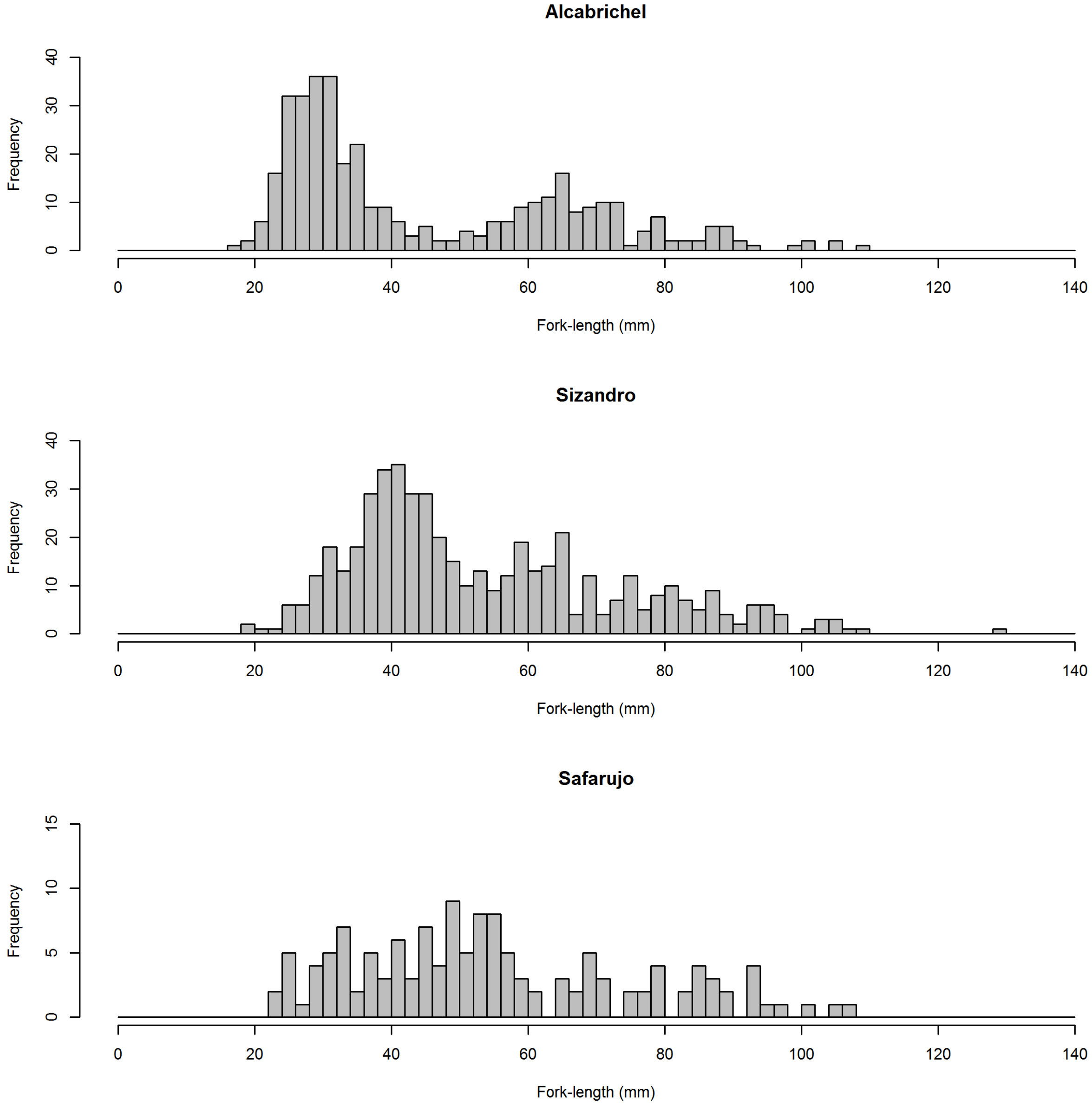


Figure 4.4. Length frequency distributions of *A. occidentale* in persistent pools in the Alcabrichel, Sizandro and Safarujo rivers. Size class amplitude is 2 mm. Note of caution: the scale in the ordinate differs among populations.

There were significant variations in fork-length among rivers (H=70.209, p<0.000) with fish from the Sizandro and Safarujo populations being larger than those from the Alcabrichel population (Dunn post *hoc test*, p<0.000) - see **Figure 4.5**.

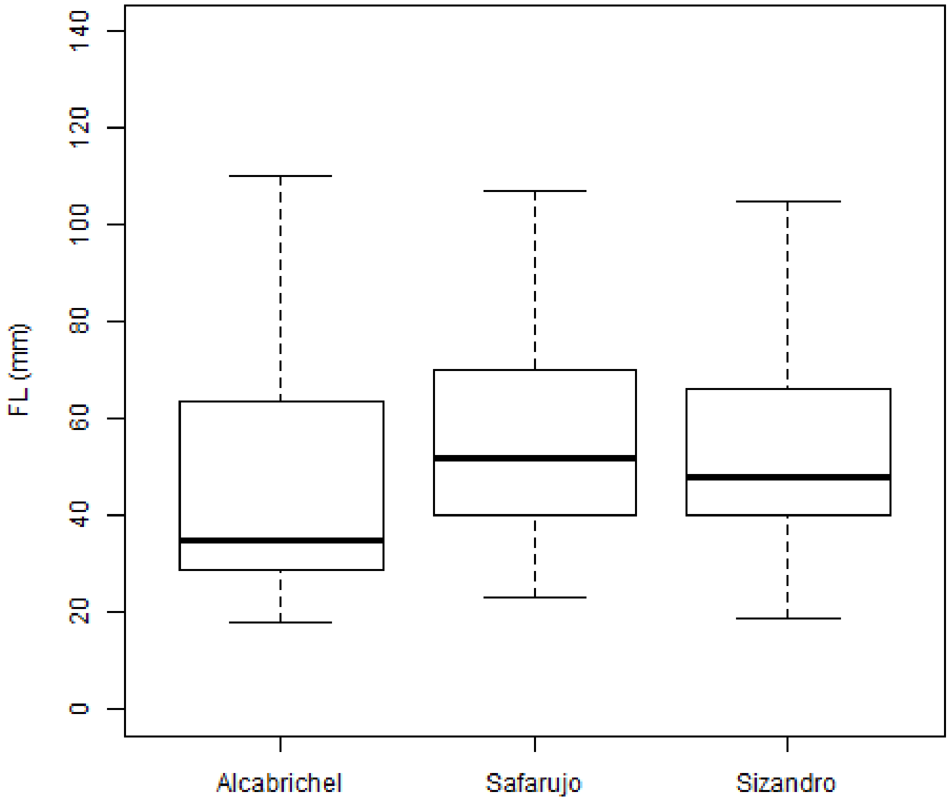


Figure 4.5. Boxplot for fork-length (FL) in wild populations, with the median marked in bold and the first and third quartiles as whiskers.

4.4. Fish condition in wild populations

Fish condition, estimated by the scaled mass index, also showed significant variations among rivers ($H=63.178$, $p<0.000$), with fish from the Sizandro (SMI = 9.90 ± 3.15) and Safarujo (SMI=11.73 ± 3.43) populations having a significantly higher condition than those from the Alcabrichel (SMI = 7.88 ± 2.81) - Dunn test, $p<0.05$.

Two of the highest mean SMI values were obtained for pools where restocking actions took place: ALC1 and SIZ1, respectively, with SMI = 4.91 and SMI = 4.75 (**Table 4.5**). The third location where fishes bred in captivity were released (ALC2), however, showed the lowest mean SMI value among all the pools sampled (SMI = 0.74, see **Figure 4.6**).

Table 4.5. Mean SMI ± standard deviation (SD), range (minimum-maximum) and number of sampled individuals (n).

Pool	Mean SMI ± SD	Range (min-max)	n
ALC1	4.75 ± 4.78	0.35 - 16.57	14
ALC2	0.74 ± 1.20	0.11 - 8.56	176
ALC3	1.51 ± 2.14	0.12 - 6.71	25
ALC4	2.63 ± 3.16	0.12 - 10.64	40
ALC5	4.86 ± 3.80	0.12 - 18.21	51
ALC6	3.33 ± 1.81	0.12 - 8.71	69
SAF1	2.90 ± 3.33	0.13 - 11.30	31
SAF2	0.56 ± 0.27	0.24 - 0.90	10
SAF3	3.68 ± 4.09	0.57 - 13.54	9
SAF4	3.88 ± 4.29	0.36 - 16.91	42
SAF5	3.59 ± 2.45	1.17 - 13.62	43
SIZ1	4.91 ± 4.17	0.14 - 22.74	38
SIZ2	4.16 ± 3.82	0.13 - 16.56	185
SIZ3	1.72 ± 0.14	0.25 - 7.53	271

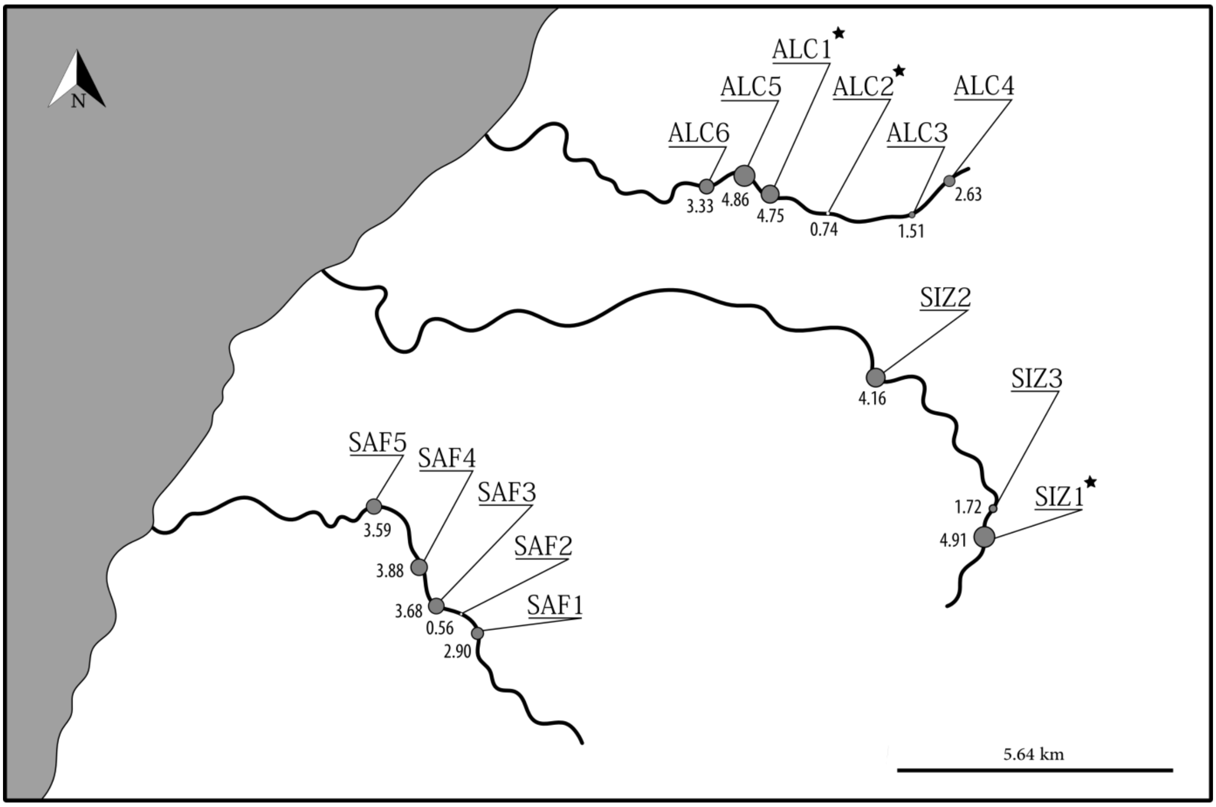


Figure 4.6. Mean scaled mass index (SMI) values for *A. occidentale* in persistent pools in the middle and upper courses of the Alcabrichel, Sizandro and Sarafujo rivers. Grey circles are proportional to the mean value of SMI in each pool. *pools where fish were released during restocking actions conducted prior to sampling.

Variation in SMI was found to be independent of PC1 (R^2=0.11, F=1.535, p<0.239), PC2 (R^2=0.01, F=0.156, p<0.699) and PC3 (R^2=0.20, F=2.923, p<0.113).

4.5. Age and growth of fish in wild and captive populations

In wild populations, fish were aged from 0+ (born in 2014) to 4+ (born in 2009) and, in all persistent pools, 0+ individuals were the most abundant, except in ALC 6 (1+). Fish aged 5+ were only found in the captive populations of Alcabrichel and Safarujo kept, respectively, at the Campelo Station and Vasco da Gama Aquarium (see **Table 4.6**).

The highest annual increment in fork-length was attained on the first year of life in all populations (**Table 4.6**). Back-calculated length at age 1 varied significantly among rivers in wild with all populations ($F=143.75$, $p<0.000$), with all populations differing from each other (Tukey *post hoc* test, $p<0.05$). Among captive populations, there were also significant differences ($F=13.99$, $p<0.000$), but only the Sizandro population was significantly different from the Alcabrichel and Safarujo specimens (Tukey *post hoc* test, $p<0.05$). Fish from the Sizandro population showed the highest mean fork-length at age 1 (FL = 72 ± 2 mm) and those from the Alcabrichel the lowest value at the same age (FL = 56 ± 1 mm) - see **Table 4.6**.

When comparing wild and captive populations of the same river, significant differences were found for all rivers. Individuals were significantly smaller in wild populations of the Alcabrichel ($T=-6.273$, $p<0.000$) and Safarujo ($T=-4.262$, $p<0.000$) than in their respective captive populations, whereas for the Sizandro individuals of the captive population were smaller than the wild ones ($Z=-4.805$, $p<0.000$).

Table 4.6. Variation in back-calculated fork-length (FL) (mean±SD) at ages 1 to 5, and annual increment (mm) in length, for wild populations from the Alcabrichel, Sizandro and Safarujo rivers and for their corresponding captive populations kept at the Campelo and Vasco da Gama Aquarium (AVG) facilities.

Anulli	1		2		3		4		5	
	n	**FL (mm)**	**n**	**FL (mm)**	**n**	**FL (mm)**	**n**	**FL (mm)**	**n**	**FL (mm)**
Alcabrichel	80	56 ± 1	28	69 ± 1	13	81 ± 1	4	94 ± 3	-	-
Annual increment (mm)	**39**		**13**		**12**		**13**		**-**	
Alcabrichel Campelo	39	60 ± 1	29	78 ± 1	16	102 ± 2	13	116 ± 3	5	131 ± 3
Annual increment (mm)	**40**		**18**		**23**		**15**		**14**	
Sizandro	16	72 ± 2	6	87 ± 3	1	102	1	128	-	-
Annual increment (mm)	**52**		**16**		**15**		**26**		**-**	
Sizandro Campelo	11	65 ± 1	3	86 ± 1	3	101 ± 4	1	119	-	-
Annual increment (mm)	**42**		**21**		**15**		**18**		**-**	
Safarujo	20	60 ± 2	13	73 ± 2	5	85 ± 2	1	96	-	-
Annual increment (mm)	**45**		**13**		**14**		**13**		**-**	
Safarujo AVG	30	64 ± 1	8	81 ± 2	6	102 ± 2	5	122 ± 4	2	133 ± 1
Annual increment (mm)	**50**		**17**		**21**		**20**		**11**	

4.6. Ethogram of reproductive behaviours

Four out of the five behaviours described in Pereira (2007) were also found during the period of preliminary observations:

- **Follow** - a male follows a female with his snout close to her tail fin or parallel to its body, without touching her and with no alterations on the movement of both individuals;
- **Chase** - a male pursues an evading female;
- **Touch** - a male follows a female with his snout close to her tail fin or parallel to its body, touching her in the ventral zone;
- **Pressure** - one or more males try to push a female to the bottom of the tank.

During this period, egg release was visualized only once, on 2nd April 2015, near a plant pot. After being pressed against the ground of the tank by several males, the spawning female started shaking its body and released several eggs. It was not perceptible if the eggs adhered to the plants, as the angle caught by the camera was insufficient. Moreover, the fixed placement of the underwater camera prevented the video recording of other egg laying events and of complete pre-spawning and spawning ethological sequences. Fish larvae were first detected on 9th May 2015.

4.7. Quantification of reproductive behaviours

A total of 77 events of the described reproductive behaviours were observed in 35 of the 90 recording sessions. In the remaining 55 sessions, no reproductive behaviours were recorded. Most of the behaviours occurred in the proximity of plants (53 out of 77) and during the 11h30-14h30 sampling period (38 out of 77) - see **Table 4.7**.

Table 4.7. Absolute frequencies of the reproductive behaviours *per* substrate and sampling period. Only frequencies marked in bold were considered for the chi-square test of independence (≥5).

Substrate/sampling period	8h30-11h30	11h30-14h30	15h00-18h00	Total
Plants	2	**29**	**22**	53
Wool spawning mops	2	**7**	**11**	20
Gravel	0	2	2	4
Total	4	38	35	77

The most frequent behaviour was “Chase” in plants and gravel substrates, and “Chase” and “Follow” in wool spawning mops (**Table 4.8**).

Table 4.8. Absolute frequencies of reproductive behaviours *per* substrate.

Substrate/behaviour	Follow	Chase	Touch	Pressure	Total
Plants	14	22	11	6	53
Wool spawning mops	8	6	5	1	20
Gravel	1	2	1	0	4
Total	23	30	17	7	77

When performing the chi-square test of independence, the 8h30-11h30 time period was excluded from the analysis due to frequencies lower than 5 (see **Table 4.7**). The absolute frequency of the observed behaviours was found to be substrate-dependent (χ^2=7.904, p<0.005), but independent from the sampling period (χ^2=0.055, p<0.815).

The peak of fish activity was on 8th May, with 35 behaviours recorded. The data logger records show that, considering the period of spawning behaviour quantification (5th May to 24th May 2015), the second highest temperature (23.13ºC) and the third highest light quantity (34157 lux) were registered on 8th May - see **Figure 4.7**). However, no significant association was detected between the frequency of behaviours and temperature and light (**Table 4.9**).

Table 4.9. Linear regression statistics (R^2, F-statistics and *p*-value) between temperature/light *per* sampling period and the frequency of behaviours *per* day.

Variable	8h30h-11h30	11h30-14h30	15h00-18h00
Temperature	R^2=0.00; F=0.019 p<0.894	R^2=0.01;F=0.146 p<0.711	R^2=0.17; F=0.203 p<0.185
Light	R^2=0.24; F>3.220 p<0.103	R^2=0.00;F=0.059 p<0.813	R^2=0.00;| F=0.023 p<0.882

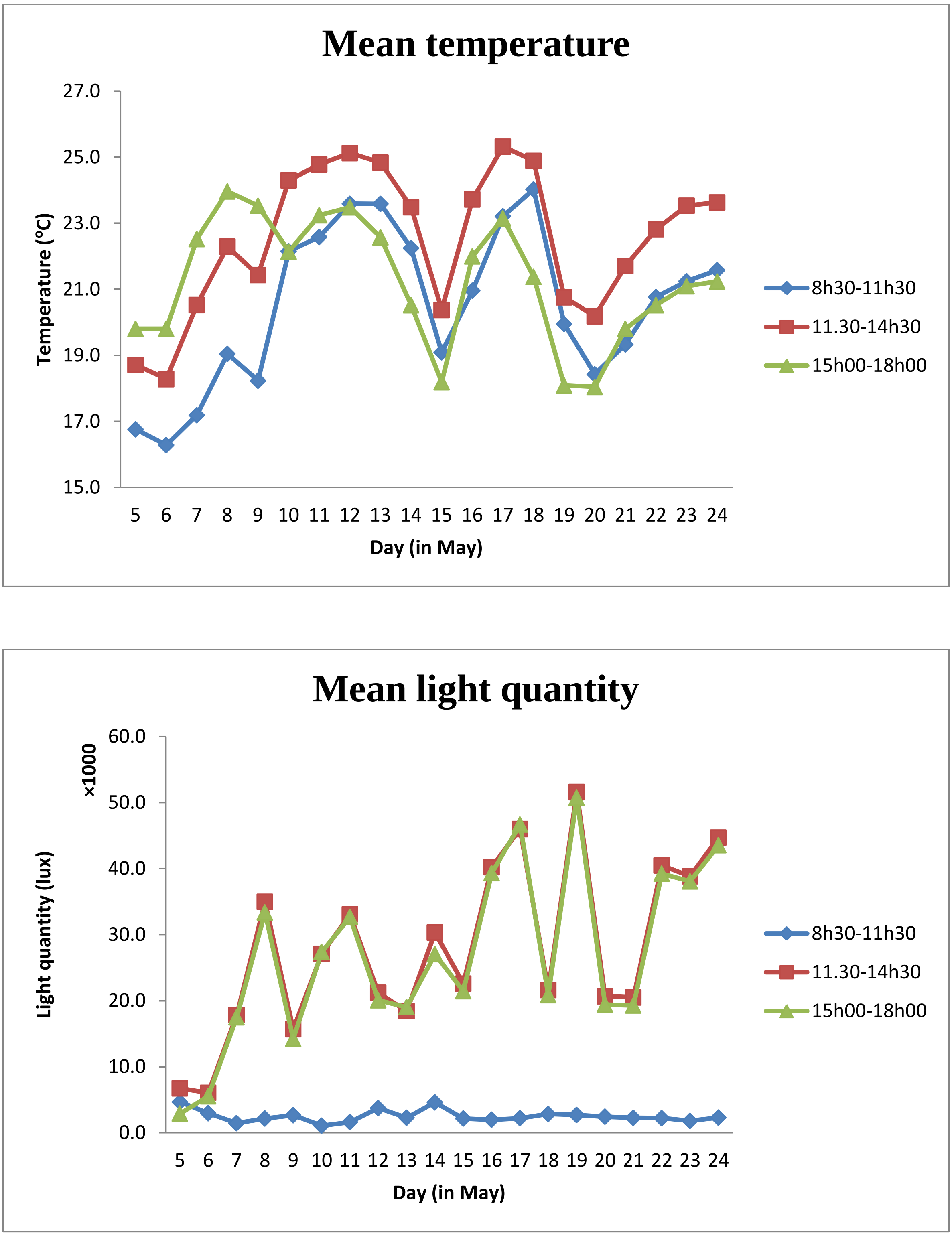


Figure 4.7. Mean temperature (ºC) and light quantity (lux) *per* block along the quantification of reproductive behaviours period (5th May 2015 and Day 10 to 24th May).

5. DISCUSSION

The combination of deterministic and stochastic factors that negatively affect species survival may result in their extinction (Frankham 2005). Often the impact of these factors is evidenced by considerable reductions of the effective population size and low levels of genetic diversity (*e.g.* Hedrick 2001, Blomqvist *et al.* 2010, McCusker and Bentzen 2013). To overcome this problem and, consequently, minimize the risk of extinction, threatened populations should be reinforced since demographic, rather than genetic factors, may be of more immediate importance in the short term (Caro and Laurenson 1994, Caughley 1994, Brook *et al.* 2002). Indeed, it is argued that populations that are declining rapidly or that are already small sized are more prone to destabilization due to interacting stochastic processes, such as unbalanced sex ratios, unstable age class distributions or habitat loss, than larger populations (Lacy 2000, Brook *et al.* 2002). The reduction of the individual fitness due to small population size or low density (*Allee effect*) is frequently a problem for endangered species and, thus, restocking can be used to provide higher long-term chances of survival (Griffith *et al.* 1989, Champagnon *et al.* 2012).

These theoretical expectations supported the enhancement of endangered populations of *A. occidentale* with captive bred individuals, in order to minimize their current risk of extinction in the wild. The data gathered in this thesis add to this view, and generally highlight that restocking already had some positive effects on wild populations of this species.

The severe drought that occurred nearly after the description of *A. occidentale*, in 2005-2006 (García-Herrera *et al.* 2007), and precipitated the implementation of its *ex situ* conservation as a safeguard measure (Sousa-Santos *et al.* 2014a), prevented the establishment of strong baseline data on the abundance of this species throughout its distribution range. Nevertheless, pilot surveys conducted in 2006 indicated that populations were small and fragmented, with the number of fish *per* summer pool in the Alcabrichel and Sizandro rivers never exceeding 40 individuals and no fish being found across the main course of the Safarujo River (C. Sousa-Santos *unpublished data*). Contrastingly, during this study, much higher numbers of individuals *per* pool were found in the Alcabrichel and Sizandro rivers, likely reflecting the restocking actions conducted in 2011 and 2013 for population reinforcement purposes. *A. occidentale* was also found in the Safarujo river, though in lower abundances, a positive sign considering it had been considered to be extinct after the 2005-2006 severe drought (Freyhof and Kottelat 2008, C. Sousa-Santos *unpublished data*).

These signs of population increment and recovery must, however, be taken with caution, since the desirable positive effect of restocking actions, habitat enhancement and water quality management must be evaluated in the medium and long term to account for the natural oscillations of abundance associated, for instance, with annual hydrological conditions as found for other cyprinids endemic to Iberian streams (*e.g.* Magalhães *et al.* 2007).

5.1. Use of summer refugia

Despite 2014 was a wet year with a "normal" hydrological summer (IPMA 2014, IPMA 2015), summer refugia in the middle and upper courses of the Alcabrichel, Sizandro and Safarujo rivers were restricted to a few persistent pools, generally with less than 70 m^3. There was considerable variability in the number of fish congregated in each pool, suggesting that some pools may act as the main sources for river recolonization after the drought and should therefore be considered as potential locations for further restocking actions. This seemed to be particularly the case for pools ALC2 and SIZ3 in the Alcabrichel and Sizandro rivers, respectively, where more than 170 individuals were found, and generally in good condition.

Overall, associations between fish and habitat conditions found in this thesis suggest that during normal hydrological years, *A. occidentale* wild populations may concentrate on shallower pools with high macrophytes' coverage, similarly to what was assessed in other fish species (Dibble *et al.* 1997, Oscoz 2006). The obtained positive association with water temperature is most likely circumstantial, since values for this variable were collected in distinct periods of the day, in different dates. Contrastingly, the positive association of fish relative abundance with nitrates highlights that refugia more prone to harbour fish may also be heavily polluted. As such, during extreme droughts, these pools might configure precarious and unstable refugia for fish, as reduction in water persistence or slight deterioration of water quality through increased nitrates and/or decreased dissolved oxygen will likely imply massive fish loss.

5.2. Length and age structure of wild populations

Besides its direct effect on increasing the size of wild populations, indirect effects of the restocking actions may include (i) the increment of population recruitment (released mature fish will contribute with their offspring to forthcoming generations); and (ii) a shift on the previous distribution of age classes. Regarding the first indirect effect, the absence of previous baseline data on the number of juveniles of *A. occidentale* produced *per* reproductive season prevents its assessment. Contrastingly, data collected during this study provide highlights regarding the effect of restocking actions on the distribution of age classes in wild populations.

As the restocking of wild populations consisted in the release of hundreds of individuals 1+, 2+ and 3+ (as a result of three consecutive generations in captivity), it was expected that, after one year, the restocked populations will not only show higher than expected frequencies 2+, 3+ and 4+ individuals, but also of 0+ and 1+ individuals, as a result of the reproduction of the released mature fish. The bimodal length distributions obtained for the Alcabrichel and Sizandro populations seemed to corroborate this hypothesis. Indeed, the combination of the obtained length frequency distributions and the back-calculated fork-length at ages 1 to 5 showed that 0+, 1+ and 2+ fish dominate the Alcabrichel and Sizandro populations. As the last and more expressive (in terms of the number of fish released) restocking actions were conducted in the Spring of 2013, mostly with F1 and F2 fish born in 2011 and 2012, it was expected that in the Fall of 2014 these fish (belonging to the age classes 1+, 2+ and eventually 3+ if the third annuli had already been deposited) and their offspring (0+ and eventually 1+) contributed to a high frequency of age classes 0+ to 2+ in wild populations, as seemed to be the case.

Although only a low number of individuals bred in captivity were identified in the wild, this may at least be partially associated with difficulties in identifying post-cut regenerated fin rays in small cyprinids (Gosline 1978) resulting in underestimation of previously marked fish. The number of recaptures may also have been underestimated due to the fact that released individuals smaller than 50 mm fork-length were not marked. In these circumstances, it seems plausible to admit that the largest mean fork-lengths and the highest condition values (SMI values) obtained for the restocking sites at the Alcabrichel and Sizandro populations are at least partially related to the release of hundreds of captive bred fish at those sites. Future restocking actions should involve more efficient individual marking (*e.g.* visible implant elastomer tags) to improve the estimation of recapture rates.

Regarding the summer refugia of the Safarujo River, the relative abundances of *A. occidentale* were lower than that of the Alcabrichel and Sizandro populations, although showing a high representativeness of small fish. This suggests that the population of the Safarujo may be naturally, slowly recovering and that recruitment may be at place (see below). As the Safarujo River was only restocked in 2015, after the field work conducted during this study, future surveys are needed to clarify if this population reinforcement can also contribute to an increase in frequency of young, small fish.

5.2. Age and growth patterns

Scale reading analysis provided baseline data on age and growth of wild and captive populations of *A. occidentale*, which was unknown until the time of this work. The annuli counts revealed that the more aged specimens from wild populations were 4+ and that the oldest specimens were found in captive populations (5+). Mean back-calculated lengths and annual increments at age *i* were also higher in captive than in wild populations of the Alcabrichel and Safarujo rivers. Contrastingly, wild specimens from the Sizandro population showed the highest mean back-calculated lengths and annual increments at age *i* among all studied populations.

Fish maintained in captivity are most likely under much less stressful conditions than those in their degraded natural habitats (*e.g.* Sousa-Santos *et al.* 2014a). This seems to justify the higher growth of the Alcabrichel and Safarujo specimens in captivity and the higher fish condition values registered in two of the three persistent pools that were target of restocking actions. However, it does not explain why wild individuals from the Sizandro grew more than the captive ones from the same population. One possible reason relies on the lower temperatures found in the Campelo Station, where those captive individuals were maintained, as it is known that fish growth is generally strongly associated with temperature (*e.g.* Handeland *et al.* 2008). The results obtained for the Sizandro wild and captive populations must, however, be analysed with caution since the sample sizes were small.

5.3. Reproductive behaviour

The observed four reproductive behaviours were already described by Pereira (2007) as pre-spawning behaviours, which provided support to the observational data presented herein and allowed further quantitative analyses. A fifth behaviour described by Pereira (2007) as “female plays dead” was not observed during this study, which may be explained by its low frequency of occurrence (reported by the author).

As Pereira (2007) conducted her observations with a captive group of 14 individuals from the Sizandro population, kept in a 90 L aquarium, the identification of the same reproductive behaviours also show that (i) *A. occidentale* shows a fixed pre-spawning ethological repertoire; (ii) this repertoire is independent of the scale used, as it is similar whether the focal group of mature adults is reduced and kept in a small aquarium (as in Pereira 2007) or if the group is composed of hundreds of individuals kept in a large tank (as in this thesis); and (iii) visual cues will likely be important as female chasing and following by males are the most common components of the pre-spawning sequence.

The highest frequencies of the observed reproductive behaviours, in all sampling periods, were attained in plants, confirming that these may act as the preferred substrate for spawning, as suggested by Pereira (2007). This information highlights the importance of accounting for aquatic vegetation when selecting future restocking sites, with persistent pools with abundant aquatic vegetation likely displaying higher availability of proper substrate for spawning.

Temperature and light quantity effects were also evaluated, because both can influence reproductive activity (King 1998 *et al.* 1998, Boeuf and Le Bail 1999), namely by being important for gonad maturation (Sousa-Santos *et al.* 2014b). Although no significant associations were found between these two factors and the total frequency of reproductive behaviours *per* day, it was evident that the peak of reproductive activity was attained after a significant increase of temperature and light, from 7th to 8th May 2015. Whether other external factors may also influence the onset of the reproductive activity is still to be evaluated.

Although important behavioural data towards the conservation of *A. occidentale* were collected, there were two constraints in this study that should be circumvented in further studies. Specifically, the large volume of the tank (3120 L) did not allow the video recording of the whole study area by the underwater camera. Also, the option for placing the camera at fixed sites, although necessary to avoid disturbance and minimize fish stress, prevented the recording of complete reproductive sequences and spawning events which would have been possible to capture if the camera was displaced to follow the focal group of fish. Indeed, spawning was only visualized once, during the trial observations, and although it was clear that the female abdomen was pressured against the ground (a behaviour herein described as "Pressure") immediately before the spawning took place, *A. occidentale* females are also expected to spawn without being previously pressured to a rigid surface (Pereira 2007). The presence of a significantly high number of juveniles in the tank may also have led to an underestimation of reproductive behaviours, which were only quantified as such when performed by clearly recognized mature males and females.

Future studies should be conducted in a tank with observation windows that would allow for the displacement of the camera by a hidden observer, to follow up the focal group of fish. This experimental setup will most likely provide additional data on the complete reproductive sequences that culminate in spawning and, more important, on male-female interactions during the courting, pre-spawning, spawning and post-spawning periods.

5.4. Final remarks and considerations for the future

The specific objectives that were put forward for this thesis were accomplished and the obtained results may be summarized as follows:

i) Summer refugia for *A. occidentale* consisted in a few small persistent pools in the middle and upper courses of the Alcabrichel, Sizandro and Safarujo rivers. The macrophytes' coverage was positively associated with fish relative abundance in summer refugia; as summer droughts increase the risk of fish loss, the identification and characterization of summer refugia used by this species provided fundamental baseline data to support future decisions and priorities regarding the implementation of habitat restoration measures and restocking actions.
ii) Along with a population increment, restocking actions conducted at the Alcabrichel and Sizandro rivers also seem to be responsible for a high representativeness of younger age classes in the wild populations.
iii) Fish raised in captivity seem to attain older ages, higher condition, larger mean back-calculated lengths and higher annual increments at age *i* than in the wild.
iv) The reproductive behaviour of *A. occidentale* involves female chasing by males, highlighting the eventual importance of visual cues, and pre-spawning activity occurs preferably on aquatic plants, independently of the period of the day.

It is crucial that the supportive breeding of endangered species for restocking purposes is complemented with *in situ* conservation of the natural habitats, so that the released fish may found favourable conditions to disperse, find food and shelter, spawn and grow to maturity. As the main goal of this thesis was to provide recommendations and guidelines for the preservation of *A. occidentale* in its natural habitats, the gathered data allowed for the establishment of some considerations that should be taken into account in future restocking and restoration actions:

(i) Since the water quality in the Alcabrichel, Sizandro and Safarujo rivers is still far from being good, some important habitat restoration and water management measures still need to be conducted in order to preserve summer refugia and, consequently, maximize the chances of recovery of *A. occidentale* populations. To improve habitat quality in the pools during the summer, it will be important to dredge sediments to increase depth; decrease the water temperature and evaporation rate by increasing instream coverage; limit water abstraction; prevent the discharge of pollutants; and set up surmountable transversal barriers to naturally improve oxygenation. Also, the eradication of *Arundo donax*, followed by the restoration of the native riparian gallery, would help stabilize the river bank, allowing the maintenance of a minimum flow level and providing shelters (*e.g.* roots of *Populus alba*) for juveniles. As dense and uninterrupted longitudinal corridors of *A. donax* prevented the access to the water course, especially in the case of the Sizandro River, their removal would also allow for the survey of other persistent pools that can act as summer refugia for *A. occidentale*. These recommendations should be taken into account in the delineation of future habitat restoration projects to avoid the uncontrolled removal of river bank vegetation, which often results in the degradation of summer refugia, including of pools that were already target of restocking actions, as was the case of ALC2 (see **Appendix VIII**);

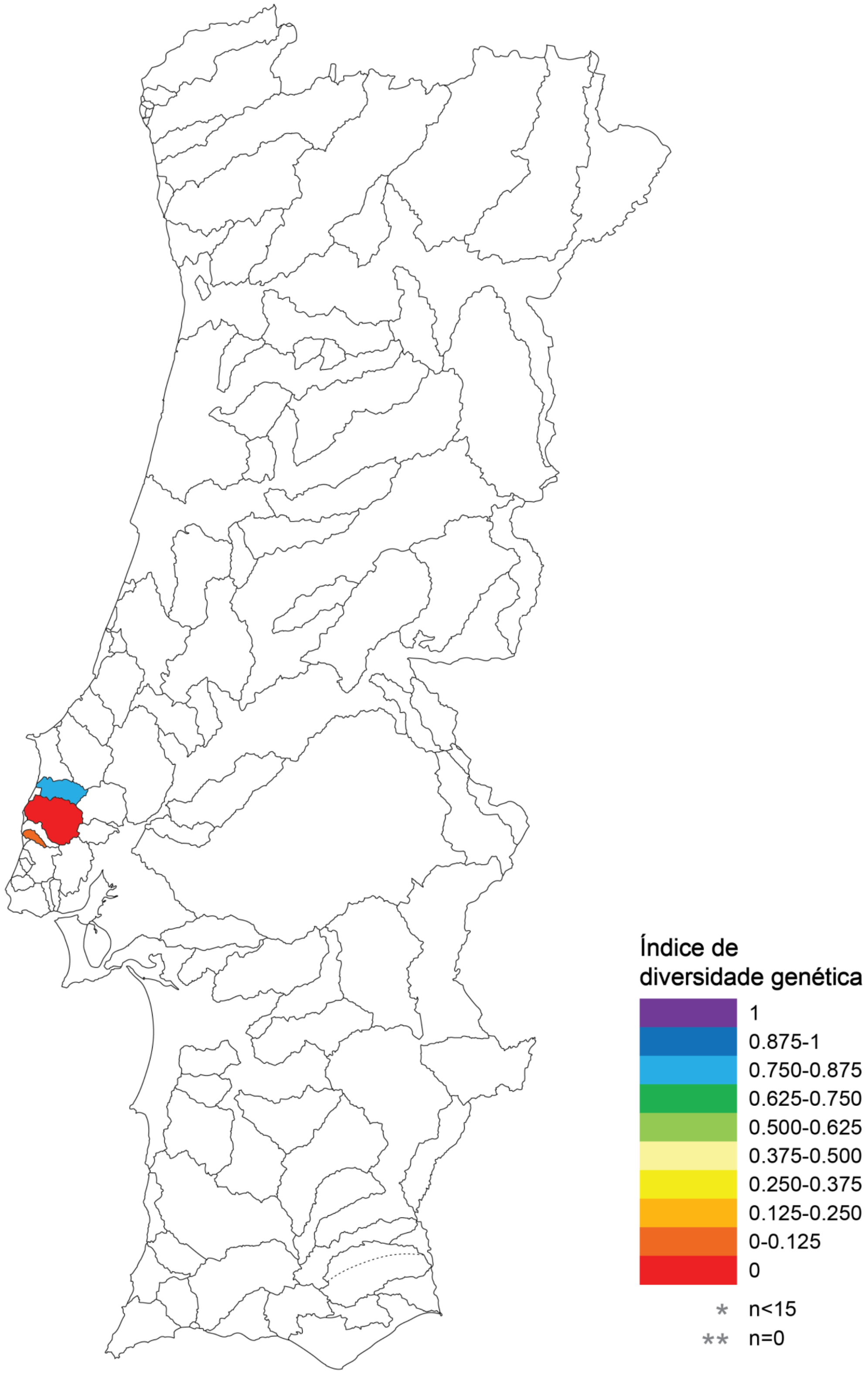
(ii) In future restocking actions, the fish to be released should be distributed by all the identified refugia, according to the water volume and abundance of aquatic vegetation available at the time in each pool. Precautions must also be taken regarding fish density in each pool, to avoid overloading pools that do not have the capacity to endure a high number of fish;

(iii) As the aquatic vegetation seems to be crucial for *A. occidentale* reproduction and population recruitment, its preservation should be promoted by environmental education actions and by providing technical assistance to the city councils in order to implement carefully designed river cleanup actions. The minimization of the erosion by the stabilization of the river banks and the preservation of aquatic vegetation will contribute to lower levels of water turbidity, which may be crucial if this species relies on visual cues for female courting by males, as suggested by the results obtained in this study.

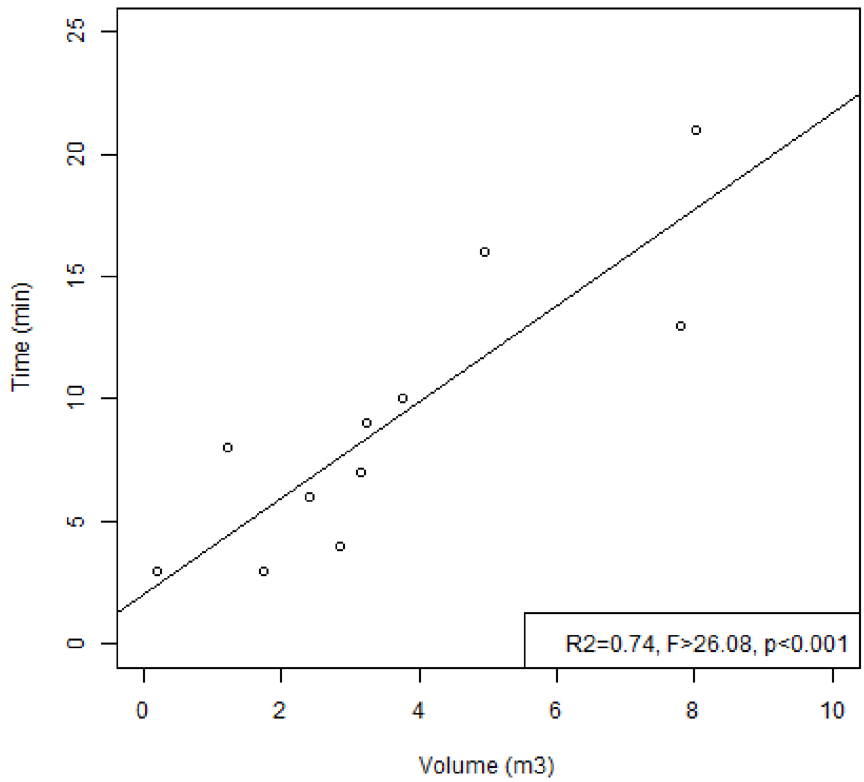
(iv) Finally, to assess the complete recovery of the three *A. occidentale* wild populations, it is important to survey fish abundance on an annual basis, in order to evaluate how the species is coping with the stochastic and deterministic stressors that may eventually compromise its survival.

APPENDICES

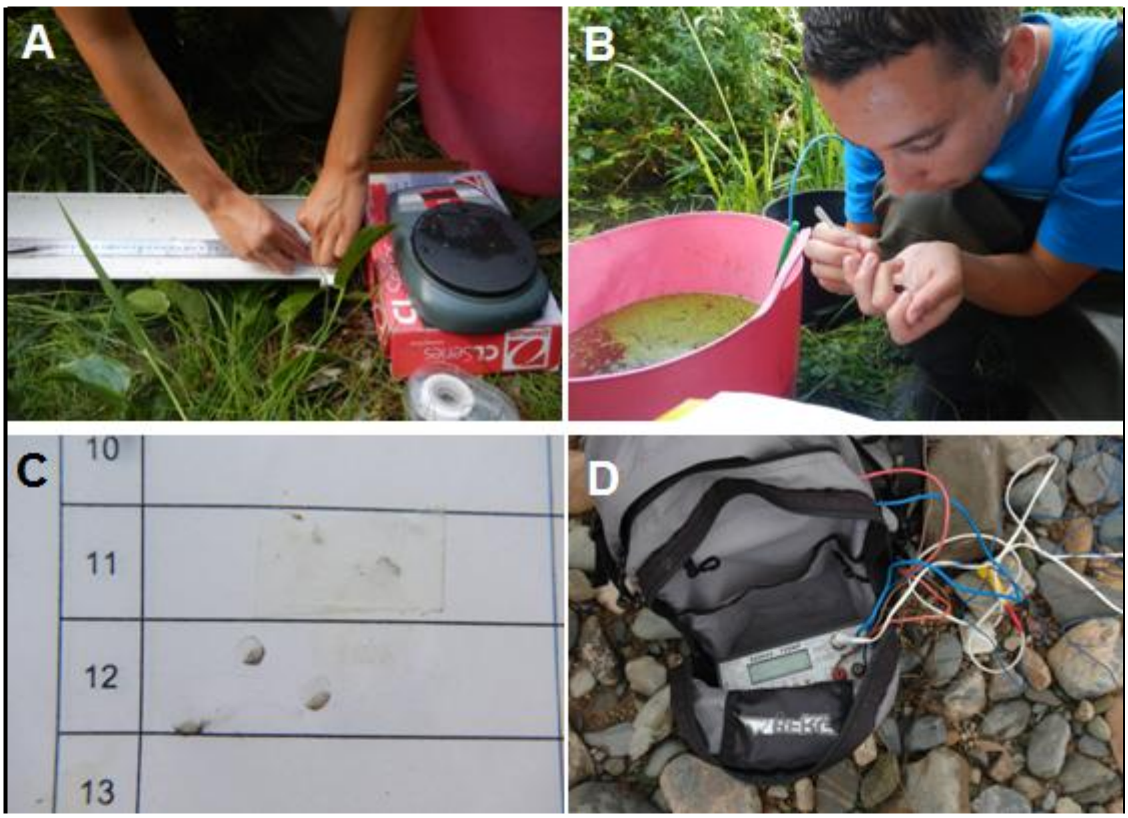
Appendix I. Genetic diversity index of the wild populations of *A. occidentale*, calculated under the scope of the FISHATLAS project (Sousa-Santos *et al.* 2013).



Appendix II. Variation in the time of fishing (minutes) in relation to pool volume (m^3).

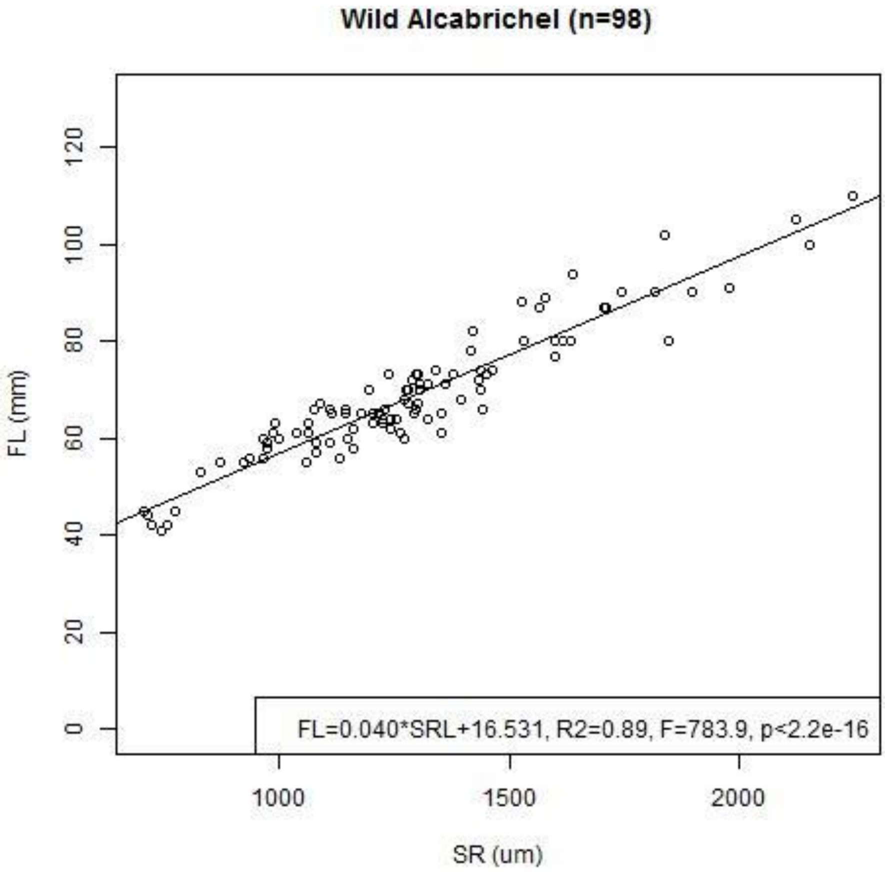


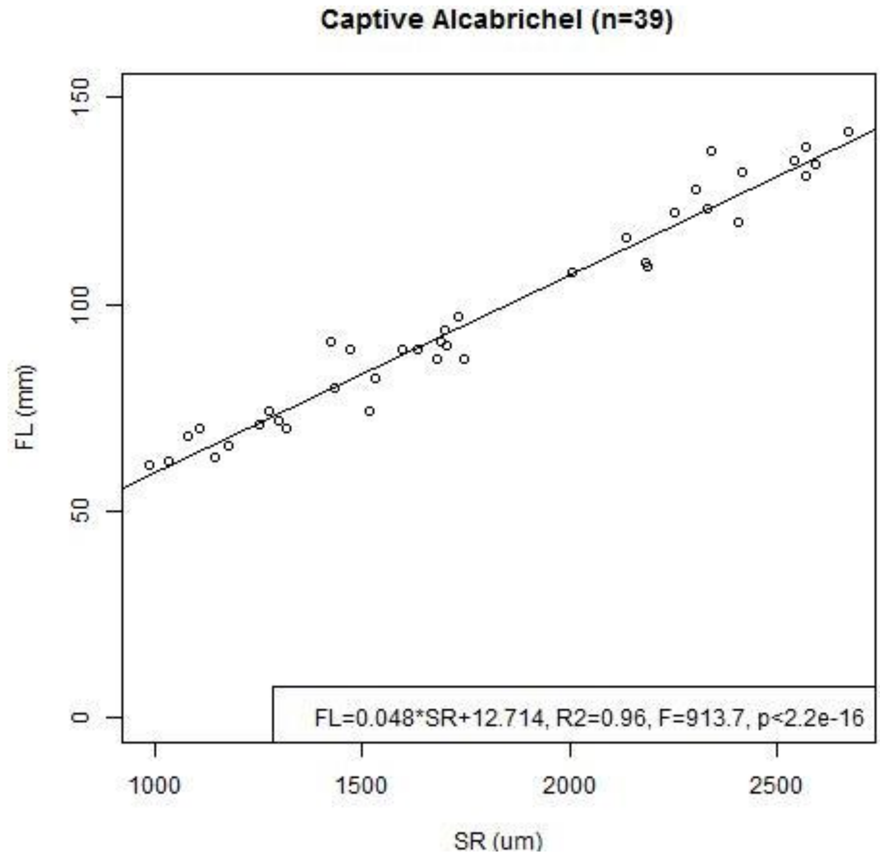
Appendix III. Field procedures used for fish sampling in persistent pools: (A) quantification of fish length and weight; (B) collection of fish scales; (C) storage of fish scales in sheets with scotch tape; (D) electrofishing device used for fish sampling.

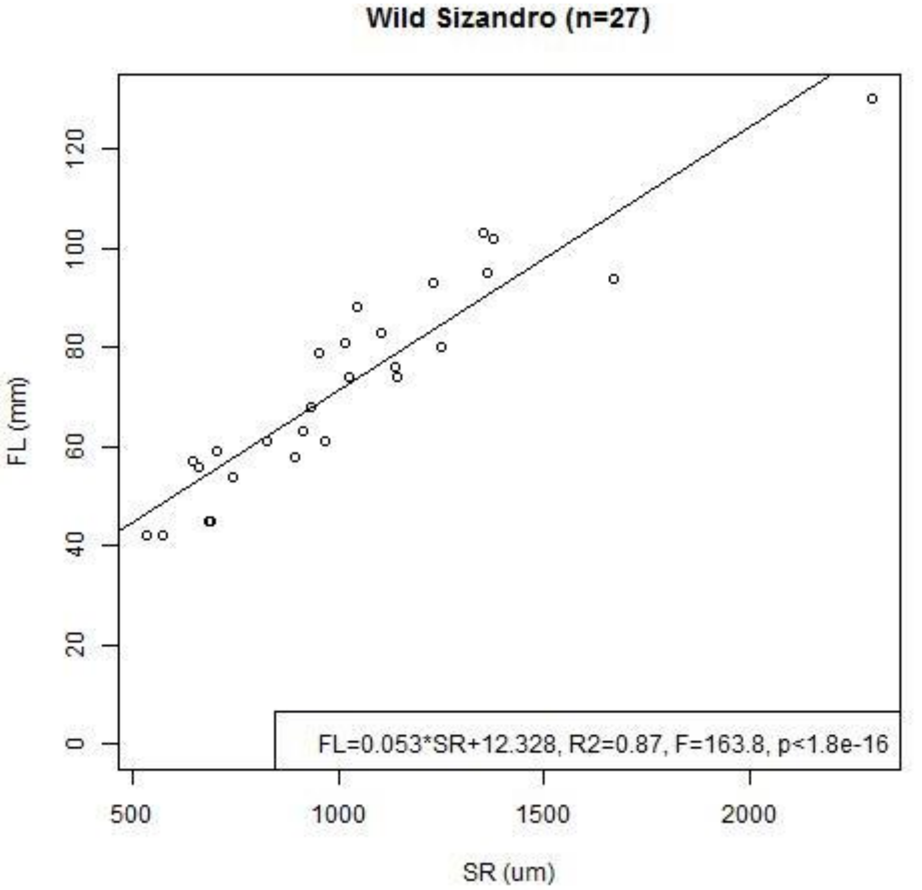


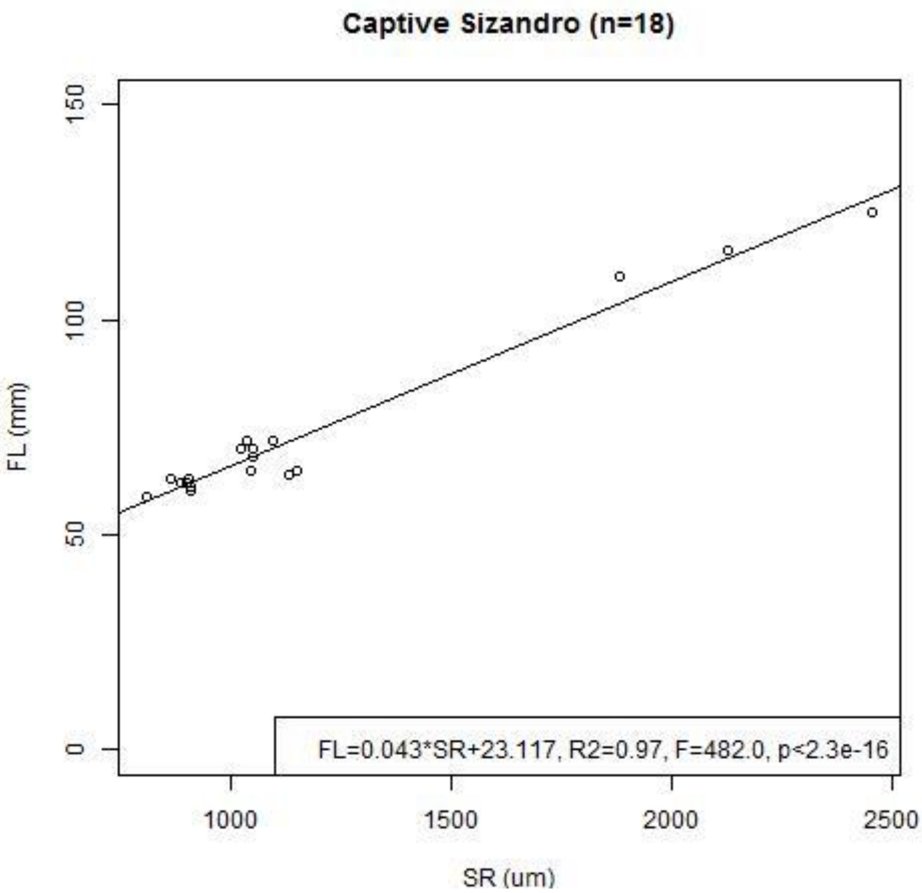
Appendix IV. Field procedures used for the assessment of habitat variables in persistent pools: (A) measuring of the percentage of dissolved oxygen, (B) kit used for measuring nitrates, nitrites, ammonia and pH; (C) scheme of the measurement of pool length (red line) and width (blue line).

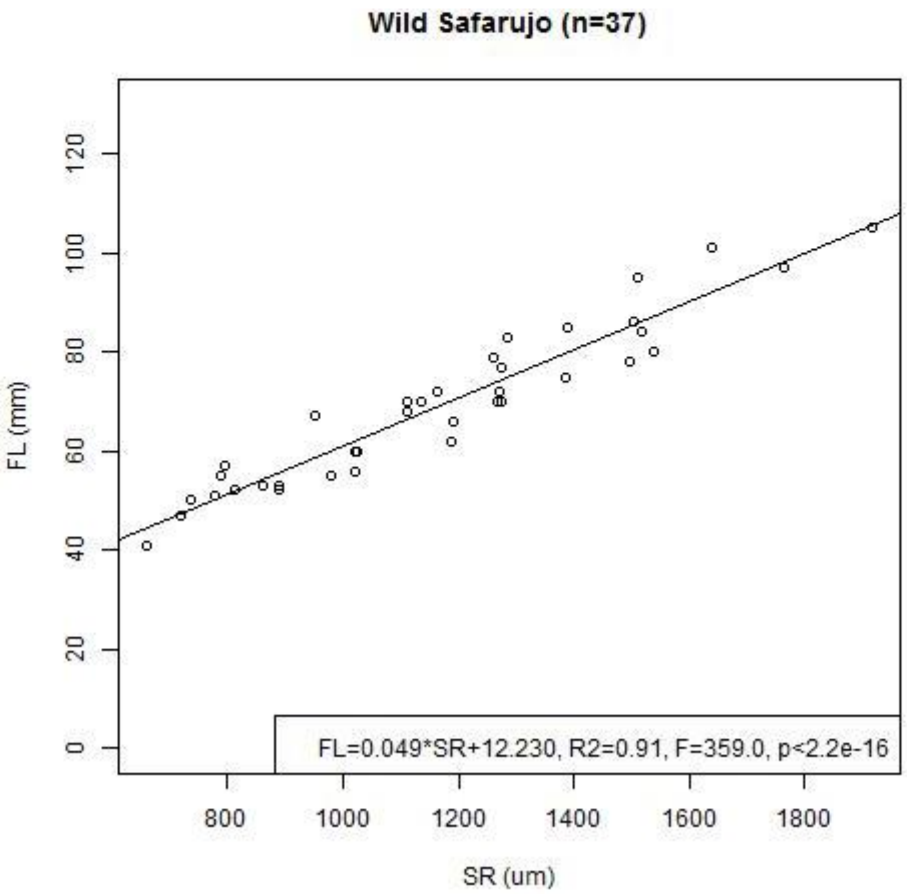
Appendix V. Linear regressions between fork-length (FL) and scale radius (SR) for wild and captive populations of *A. occidentale* from the Alcabrichel, Sizandro and Safarujo rivers, used to determine back-calculated fork-lengths at age *i*.

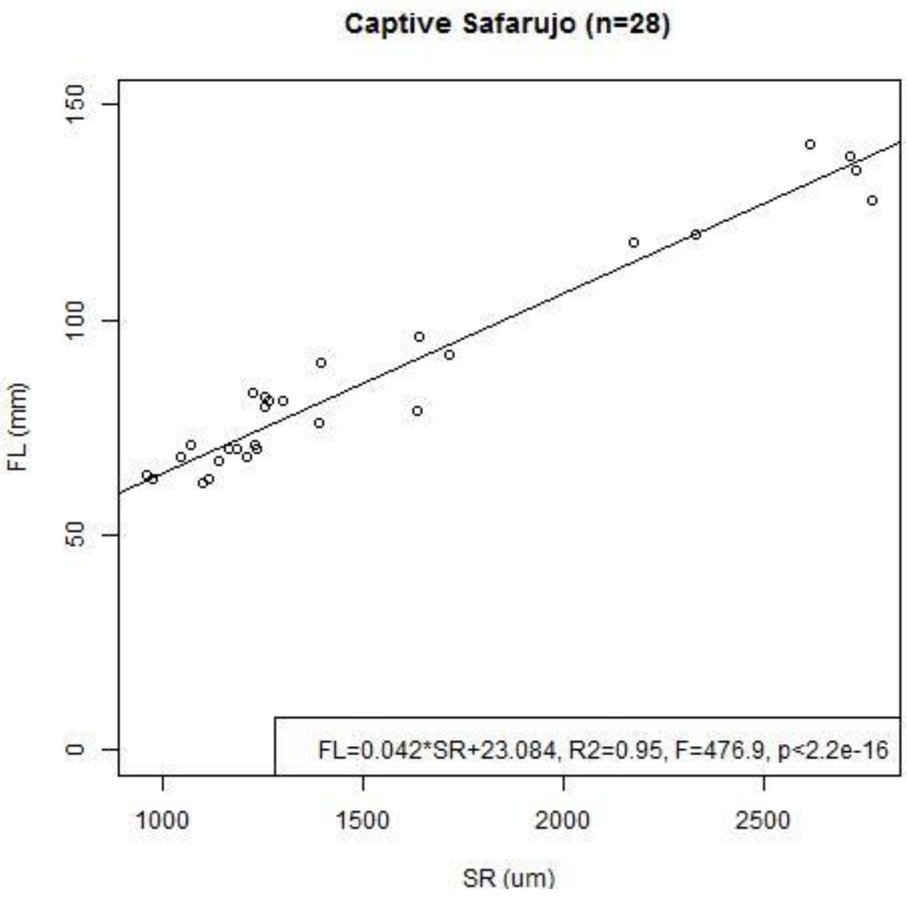





Wild Sizandro (n=27)
FL (mm)
0
20
40
60
80
100
120
500
1000
1500
2000
SR (um)
FL=0.053*SR+12.328, R2=0.87, F=163.8, p<1.8e-16


Captive Sizandro (n=18)
FL (mm)
0
50
100
150
1000
1500
2000
2500
SR (um)
FL=0.043*SR+23.117, R2=0.97, F=482.0, p<2.3e-16


Wild Safarujo (n=37)
FL=0.049*SR+12.230, R2=0.91, F=359.0, p<2.2e-16
FL (mm)
SR (um)


Captive Safarujo (n=28)
FL=0.042*SR+23.084, R2=0.95, F=476.9, p<2.2e-16
FL (mm)
SR (um)

Appendix VI. Spawning mops with adhesive eggs from *A. occidentale*, at the Vasco da Gama Aquarium. Photo: Carla Santos.

Appendix VII. Pearson correlation between habitat variables and relative abundance of fish (FISH) in persistent pools. Coefficients over 0.80 are marked in bold. Abbreviations for habitat variables are described in Table 2 (**3.Material and Methods**, section 3.1).

Variable	FISH	AREA	VOL	TEMP	O2	PLANT	SHAD	DET	DSUB	HSUB	NO2
FISH											
AREA	0.46										
VOL	0.16	**0.88**									
TEMP	0.50	-0.33	-0.52								
O2	0.26	0.07	-0.03	0.14							
PLANT	0.73	0.17	-0.09	0.43	0.17						
SHAD	-0.20	0.18	0.16	-0.42	-0.05	0.07					
DET	-0.22	0.20	0.25	-0.22	0.08	-0.19	-0.28				
DSUB	0.03	0.02	0.04	0.04	-0.45	0.05	0.36	-0.76			
HSUB	0.65	0.48	0.39	-0.01	0.31	0.59	-0.13	-0.16	0.07		
NO2	0.41	-0.01	-0.13	0.33	0.00	0.41	-0.32	0.16	-0.22	0.45	
NO3	0.41	0.10	-0.07	0.49	0.04	0.32	-0.21	0.18	-0.20	0.15	**0.81**

Append VIII. Evolution in the river bank of the persistent pool ALC2, from the time it was surveyed (September 2014) to the 3-month period (July 2015) that followed the "restoration" action promoted by the local hall (April 2015), whose goal was to remove *Arundo donax* plantations.

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